EFFECT OF ANTITHROMBOTIC DRUGS ON PLATELET FUNCTION AND RECEPTORS AND AN INVESTIGATION INTO PLATELET CHOLESTEROL

This thesis is presented to University College London in part fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

by

I. ANITA JAGROOP-DEARING

Department of Surgery, Division of Surgical and Interventional Science & Department of Clinical Biochemistry, University College London Medical School, University College London, Royal Free Hospital Campus, Pond Street, Hampstead, London, NW3 2QG, UK.

DECLARATION

I, [Indera Anita Jagroop-Dearing] _____, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Candidate: Indera Anita Jagroop-Dearing

Primary Supervisor: DP Mikhailidis BSc MSc MD FRSPH FCP FFPM FRCP FRCPath
Academic Head, Department of Clinical Biochemistry
Royal Free Hospital Campus,
University College London Medical School,
University College London (UCL),
Pond Street,
London NW3 2QG, UK

Secondary Supervisor: Professor Geoffrey Burnstock PhD DSc FAA FRCS(Hon) FRCP(Hon) FMedSci **FRS**
Autonomic Neuroscience Centre,
University College Medical School,
Rowland Hill Street,
London NW3 2PF, UK

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ABSTRACT

The aim of this thesis was to assess several aspects of platelet function, especially in response to drugs.

Peripheral arterial disease (PAD) is associated with hyperactive platelets that may be resistant to aspirin. Therefore, the effect of the administration of clopidogrel, aspirin or both anti-platelet agents was investigated in patients with PAD. Platelet function was assessed by different techniques including platelet shape change (PSC), an early phase of platelet activation that precedes aggregation. PSC was measured using a high-resolution channelyzer and expressed as median platelet volume (MPV). There was an increased inhibitory effect of clopidogrel plus aspirin on platelet activation. Clopidogrel was a more potent inhibitor of ADP-induced platelet activation than aspirin.

The ADP receptors, $P2Y_1$, $P2Y_{12}$ and $P2X_1$ influence platelet activation. The $P2Y_1$ receptor antagonist MRS 2179 blocked ADP-and ADP plus serotonin-induced PSC *in vitro*. The $P2Y_{12}$ receptor antagonist AR-C69931MX significantly inhibited ADP-induced PSC. We demonstrated for the first time that the IC_{50} of a $P2Y_1$ receptor-blocker can be derived using PSC.

Tirofiban is a glycoprotein IIb/IIIa receptor antagonist and therefore a powerful inhibitor of platelet aggregation. Tirofiban did not affect fibrinogen/agonist-induced PSC but inhibited platelet aggregation *in vitro*. A lack of inhibition of PSC may limit the use of tirofiban in clinical practice.

Current methods to measure platelet cholesterol (PC) involve complex extraction processes. A faster and simpler technique to measure PC was developed. There was a significant correlation between PC and some circulating lipid levels. This method may be useful for multiple sampling. PC may represent 'tissue' cholesterol levels. Furthermore, because high PC is associated with increased platelet activity, statins may decrease both PC and risk of thrombosis.

The findings in this thesis may provide a better understanding of platelet function and how to assess it. Furthermore, potential therapeutic targets are discussed.

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ABBREVIATIONS

AA	Arachidonic acid
ABP	Actin binding Protein
ABPI	Ankle-Brachial Pressure Index
AC	Adenyl cyclase
ACD	Acid-citrate-dextrose
ACE	Angiotensin converting enzyme
ADP	Adenosine 5'-diphosphate
ADPase	Adenosine diphosphatase
AMP	Adenosine monophosphate
ASA	Acetyl silicylic acid (aspirin)
ATP	Adenosine 5'-triphosphate
βTG	Beta-thromboglobulin
BT	Bleeding-time
Ca^{2+}	Calcium
CADP	collagen and ADP
CaI	Calcium ionophore
cAMP	Adenosine 3': 5'-cyclic monophosphate
CEPI	Collagen and epinephrine
cGMP	Guanosine 3' 5'-cyclic monophosphate
CHF	Congestive heart failure
CEPI	Collagen and epinephrine
CADP	Collagen and adenosine diphosphate
CT	Closure time
COSH	Committee For Occupational Safety And Health
COX	Cyclooxygenase
COX-1	Cycloxygenase-1
CPA	Cone and platelet analyzer

cpm	Counts per minute
Ct	Closure time
CV	Coefficient of variation
DM	Diabetes mellitus
DMS	Demarcation membrane system
DNA	Deoxyribonucleic Acid
DTS	Dense tubular system
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme immuno assay
EM	Electron immunoassay
ET	Endothelin
FACS	Fluorescence Activated Cell Sorting
fc	Final concentration
fib	Fibrinogen
fl	Femtoliters
GP	Glycoprotein
G-Protein	Guanine nucleotide-binding protein
GTP	Guanine triphosphate
HBP	High blood pressure
Hct	Haematocrite
HDL-C	High density lipoprotein cholesterol
HPLC	High performance liquid chromatography
IC_{50}	Concentration for 50% inhibition
IDDM	Insulin dependent diabetes mellitus
IHD	Ischaemic heart disease
INDO	Indomethacin
LDL-C	Low density lipoprotein cholesterol
Mg^{++}	Magnesium

MI	Myocardial infarction
MK	Megakaryocyte
MLC	Myosin light chain
MPV	Median platelet volume
NAF	Naftridrofuryl
NCEP	National Cholesterol Education Program
NIDDM	Non-insulin dependent diabetes mellitus
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
OCS	Open canalicular system
PA	Phosphatidic acid
PAD	Peripheral arterial disease
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PAU	Platelet aggregation units
PC	Platelet cholesterol
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PF4	Platelet factor 4
PGE1	Prostaglandin E1
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PI	Phosphytidylinositol
PK-A	Protein kinase A
PK-C	Protein kinase C
PMC	Platelet membrane count
PPACK	Phenylalanine-proline-arginine chloromethyl ketone
PPP	Platelet poor plasma
PRP	Platelet rich plasma

PSC	Platelet shape change
PTCA	percutaneous transluminal coronary angioplasty
RBC	Red blood cell
RFH	Royal Free Hospital
RGDS	Arginine-glycine-aspartate-serine
RIA	Radioimmunoassay
ROTEM	Thromboelastometry
RT	Room temperature
SC	Surface coverage
ScEM	Scanning electron microscopy
ScMC	submembranous cytoskeleton
SCS	surface-connected system
SEM	Standard error of the mean
SI Units	System International d'Units
SPA	Spontaneous platelet aggregation
TC	Total cholesterol
TEG	Thromboelastography
TG	Triglycerides
TPA	Tissue plasminogen activator
TRAP	Thrombin Receptor Activation Peptide
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
UDP	Uridine 5'-diphosphate
NEQAS	National External Quality Assessment Service
UTP	Uridine 5-triphosphate
VASP	Vasodilator-stimulated phosphoprotein
vWD	von Willebrand’s disease
vWF	von Willebrand’s factor
WB	Whole blood

OVERALL AIMS AND HYPOTHESIS

Aims: To gain a better understanding of the effect of antithrombotic drugs on platelet function and receptors particularly in relation to peripheral arterial disease (PAD). I also aimed to develop a rapid and simple method for the measurement of platelet cholesterol (PC), a variable known to influence platelet function.

Hypothesis: PAD patients tend to have hyperactive platelets. The usual drug prescribed is aspirin. However, some of these patients may be aspirin resistant. Thus, it is important to consider other antiplatelet drugs like clopidogrel which acts on $P2Y_{12}$ purinergic receptors. I investigated the effect of clopidogrel, aspirin and both drugs on platelet function in PAD patients. Platelet function was assessed by different techniques, including platelet shape change (PSC), an early phase of platelet activation. A channelyzer method was developed in this laboratory to measure PSC. This technique is sensitive, reproducible and has high resolution. The hypothesis was that clopidogrel was superior to aspirin and that both drugs together were superior to monotherapy.

It is known that clopidogrel acts via the $P2Y_{12}$ purinergic receptors. However, I also wanted to investigate the involvement of other platelet purinergic receptors (namely $P2Y_1$ and $P2X_1$) on PSC. Others have implicated the $P2Y_1$ purinergic receptor in PSC, but used a less sensitive method (optical transmission aggregometry). It has since come to light that these measurements actually represent the formation of platelet microaggregates rather than PSC. Thus, I quantitatively assessed PSC using MRS2179, a $P2Y_1$ purinergic receptor blocker. Because clopidogrel is a prodrug, I used the $P2Y_{12}$ purinergic receptor blocker ARC

69932IMX to assess the effect of this purinergic receptor on PSC. To my knowledge, this is the first study that actually quantifies PSC inhibition by this or any other $P2Y_{12}$ receptor blocker. I also investigated whether the $P2X_1$ purinergic receptor is/is not involved in PSC. PAD patients may be resistant to aspirin and/or clopidogrel. It is therefore important to consider antiplatelet drugs that work via other receptors. Hence, I investigated the effect of a GPIIb/IIIa receptor antagonist, tirofiban, on fibrinogen- and agonist-induced PSC and aggregation. There is evidence linking raised plasma fibrinogen levels and platelet hyperactivity with vascular events. I also assessed if fibrinogen affects PSC in human platelets. The hypothesis would be that GPIIb/IIIa antagonists like tirofiban do not affect PSC and that this may be a reason for their diminished efficacy.

It is well documented that increased platelet activity is linked to raised circulating cholesterol levels. Also, high platelet cholesterol (PC) levels affect platelet membrane fluidity which may also modulate the conformational state of platelet receptors, thereby indirectly affecting the effectiveness of antiplatelet drugs. Moreover, PC can be used as a marker of 'tissue' cholesterol. The present methods to assess PC are cumbersome. They involve time consuming and complex processes with toxic and potentially harmful chemicals. I set out to develop a simplified, reproducible, rapid and safe method to measure PC. I also investigated if there was a correlation between PC and circulating lipid levels. The hypothesis was that such a correlation would be present since others had reported a fall in PC after treatment with a statin. A rapid PC method would make it possible for me to evaluate all the receptor experiments described above in relation to PC levels. The hypothesis would be that PC levels will influence platelet receptor responses.

CHAPTER 1

General Introduction

1.1 A Brief History about Platelets

The mechanisms by which platelets are produced have been studied for over 100 years (Born, 1967a,1967b). Platelets were initially termed "the dust of the blood". It was James Homer Wright in 1906 who first recognized how giant bone marrow megakaryocytes (MKs) 'gave birth' to platelets (Kuter, 1996). Claims have been made by different scientists as to who was the first to identify platelets. From as early as 1873 Osler described their disc-like structure (Osler W, 1874). Seven years later Bizzozero (Coller, 1984) identified the platelet anatomically and related them to haemostasis and experimental thrombosis. He went on to demonstrate that platelets adhere to the blood vessel wall at the site of injury to form aggregates. In fact, Bizzozero was the first to identify bone marrow megakaryocytes but he never recognised them as precursors of platelets. Hence, James Homer Wright in 1906 used his new polychrome staining solution (Wright's stain) to show the similarities in shape and colour of the red-to-violet granules in platelets and megakaryocytes and is credited as the first to demonstrate the formation of platelets. Moreover, since the intervening 125 years since Bizzozero's pioneering work, we have seen a considerable increase in our understanding of the platelet with regard to its structure, formation and role in haemostasis and thrombosis (Born 1965, Coller, 1984).

1.2 Platelet Formation

Megakaryocytes (MKs) are highly specialised precursor cells that function solely to produce and release platelets into the circulation. Platelets sometimes known as thrombocytes along with erythrocytes (red blood cells, RBC) and leucocytes (White *et al.,*

1976) circulate in the blood. Erythrocytes account for 96% of the total number of blood cells and are responsible for carrying oxygen to cells, while leucocytes are involved in immune and inflammatory responses. An average person has approximately 5.5 litres of blood. The different cell types circulating in the blood along with their count and diameter are shown below:

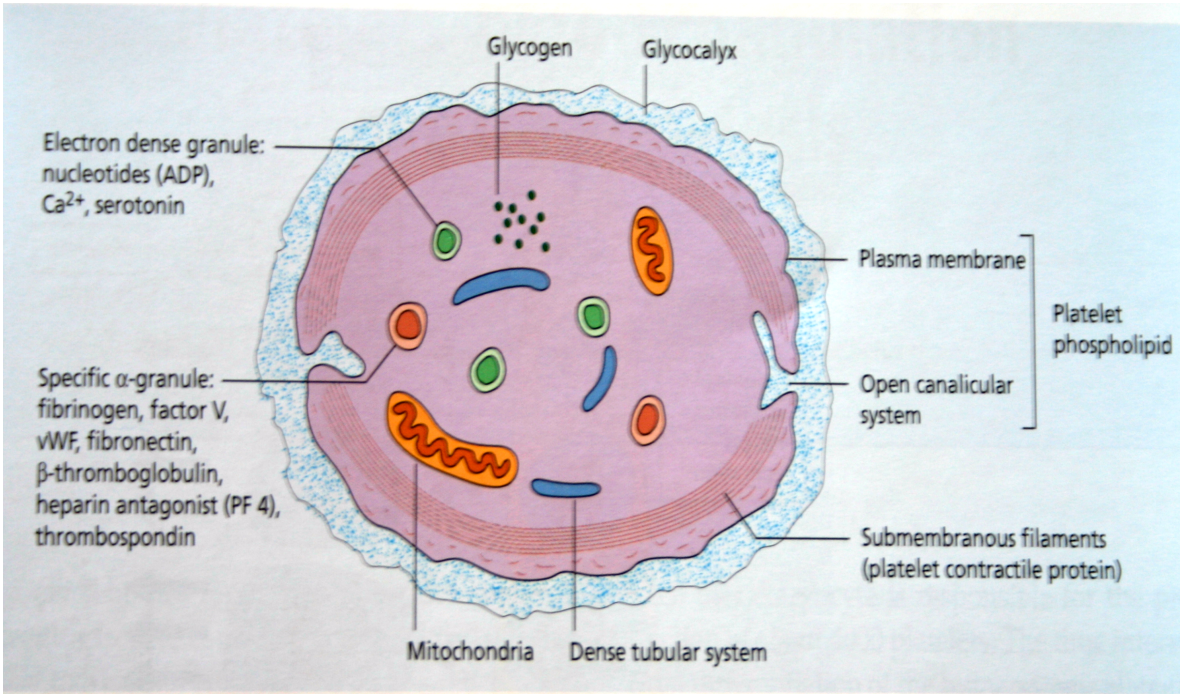
Platelets	150-450 x 10^9/L	0.5-3 μm
Erythrocytes	M 4.4 – 5.8 x 10^{12}/L F 3.9 – 5.2 x 10^{12}/L	7 μm 7 μm
Leucocytes	4.5 – 11.0 x 10^9/L	12 μm

Although it is accepted that platelets are derive from megakaryocytes (MKs), the mechanisms involved remain controversial and complex. Throughout the years, several models of platelet production have been proposed. These include: (a) platelet budding, (b) cytoplasmic fragmentation via the demarcation membrane system (DMS), and, (c) pro-platelet formation. However, the mainstream understanding is that MKs are descended from pluripotent stem cells and undergo multiple DNA replications without cell divisions, a process called endomitosis (May *et al.,* 1998). During endomitosis, polypoid MKs initiate a rapid cytoplasmic expansion phase which is characterised by the development of a highly developed DMS and the accumulation of cytoplasmic proteins and granules essential for platelet function (Hartwig *et al.,* 1991). In the final stages of development, the MKs cytoplasm undergoes a re-organisation into beaded cytoplasmic extensions called pro-platelets. Finally, the pro-platelets yield individual platelets.

1.3 Platelet Structure

The platelet has a life span of 7-10 days. The platelet plays a double role of being 'good' and 'evil'. They may be called upon to aid in host survival, but unfortunately, functions important in host preservation can also contribute to host destruction by being involved in thrombosis. Thus, an understanding of the platelet structure (see figure 1.1), pathology and physiology may prove useful in the treatment of vascular disease and bleeding disorders.

Figure 1.1 Platelet Structure



1.3.1 The Resting Platelet

A distinguishing feature of the platelet is that it lacks a nucleus normally found in most cells. Human platelets circulate in the blood as discs and are heterogenous in size with dimensions of 0.5 x 3.0 μm (Hartwig *et al.,* 1991). The average mean cell volume is 6 to 10 femtoliters (fl) (Matsuoka *et al.,* 2000). It is thought that the disc shape of the platelet (see figure 1.4) may aid in some aspect of their ability to flow close to the endothelium in the blood stream. The platelet plasma membrane is relatively smooth however high-resolution scanning electron microscopy (ScEM) (Hartwig *et al.,* 1991) suggested a folded appearance. It is thought that these tiny folds may provide additional membrane needed when platelets spread on surfaces (Stossel *et al.,* 2001).

There are periodic small openings of the surface connecting the open canalicular system (OCS), a complex network of inter-winding membranes that permeate the platelet's cytoplasm (Kovacsovics *et al.,* 1996). The OCS has two major functions, firstly to serve as a passageway to the blood stream in which the contents can be released, and secondly to function as reservoir of plasma membrane and membrane receptors. In the resting platelet at least one third of the thrombin receptors located on the OCS is awaiting transport to the surface of activated platelet. Another membrane system of the platelet is the dense tubular system (DTS) and serves mainly the calcium storage system. In the resting platelet, the DTS maintains the cytosolic calcium concentrations in the nanomolar range (see figure 1.2) (Yoshida, 1989; Ebbeling, 1992).

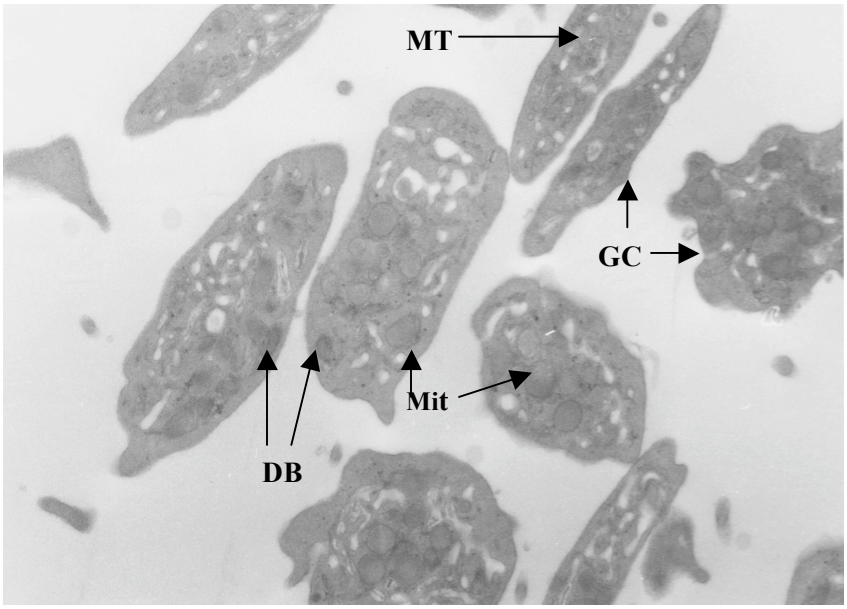


Figure1.2 Representative TEM showing resting platelets.
A transmission electro micrograph (TEM) of a representative population of normal resting platelets (see Methods, 2.6) showing various dense bodies and other structures and granules.
GC-glycocalax; Mit- mitochondria; DB-dense body

The platelet plasma membrane has a thick exterior coat called the glycocalyx. The lipid bilayer on which the glycocalyx rests serves an extremely important role in the acceleration of clotting, a function not shared by other cells in the circulating blood. When resting, the lipid bilayer of the platelet contains large concentrations of transmembrane receptors (Moake *et al.,* 1988). The most important receptors found on the surface of the resting platelet include the glycoprotein receptor for von Willebrand factor (vWF) (Moake *et al.,* 1988), receptors for adenosine diphosphate (ADP), thrombin, epinephrine and thromboxane A_2 (TXA_2); the F_c receptor $F_c\gamma$ RIIA; and the β3 and β1 integrin receptors for fibrinogen and collagen (Born 1965, 1967a, 1967b, Berridge, 1984). These receptors are known to play a key role in adhesion of platelets to damaged surfaces, and also may trigger

full activation of the platelet to promote platelet aggregation Born 1976a, 1967b) and interaction with other cellular elements, and to accelerate the process of clot formation (Matzdorff, 2005).

An interesting characteristic of the platelet is that it contains granules within which biologically active molecules are stored. In resting platelets, granules are situated close to the OCS membranes. Platelets have three major recognised types of storage granules: 'dense' granules (calcium rich and therefore opaque on electron micrography), α-granules and lysosomes (table1.1). The granules contain substances with platelet pro-aggregatory, vasoconstrictory, mitogenic and vascular permeability enhancing effects (Mininkova, 2010; Hattori, 1974).

Table 1.1: Intra-platelet Granules

α-granules	**Dense Granules**	**Lysosomes**
PDGF	Ca^{2+} Mg^{2+}	Acid hydrolases
PF-4	ADP, ATP	*β*-hexosaminidase
β-TG	Serotonin	
Fibrinogen	Histamine	
Fibronectin	Noradrenaline	
vWF	Adrenaline	

(See list of abbreviations)

Platelet α-granules can uptake and package plasma proteins via receptor-mediated endocytosis and pinocytosis. There are also other membrane proteins critical to platelet function that are packaged into α-granules; these include $\alpha IIb\beta_3$, CD62P, CD36 as well as the majority of cellular P-selectin. The contents of the dense granules (sometimes called

dense bodies), 250 nm in size, function primarily to recruit additional platelets to sites of vascular injury. These contents are released upon platelet activation initiating PSC and aggregation (both of which will be considered further in this thesis) as well as the granule release reaction. The third major storage granule type, the lysosomes, functions primarily in the break down of material ingested by phagocytosis or pinocytosis.

The technique of detergent-permeabilization followed by rapid freezing, freeze drying, and metal shadowing has been previously used to obtain high resolution images of cytoskeleton of the resting platelet (Rand *et al.,* 2003). As previously mentioned the resting platelet is disc shaped. This shape is maintained by a highly specialised cytoskeleton (Rand *et al.,* 2003). When the platelet is resting there are three major components of the cytoskeleton. Starting from the plasma membrane inward they include (1) a spectrin based skeleton, adherent to the cytoplasmic side of the plasma membrane, (2) a microtubule coil running along the perimeter of the disc, and (3) the actin filaments, a rigid cross linked network that fills the cytoplasmic space of the cell.

1.3.2 The Activated Platelet

An important function of the platelet is to detect damage on vessel walls and respond rapidly. Thus, in response to vascular damage platelets undergo dramatic changes by attaching themselves to the damaged area, changing shape and spreading over that injured site.

Initiation of activation entails (a) aggregation, (b) secretion, (c) activation of the biochemical synthetic pathway to produce and release TXA_2 and other potent platelet agonists, and, (d)

then activation of a major platelet integrin, αIIbβ3, the receptor for vWF (Harrison *et al.,* 1999). A major component of this thesis involves PSC thus, this process is explained in some detail, below.

When activated, platelets follow a sequence of complex events instigated by a rise in cytoplasmic calcium levels.

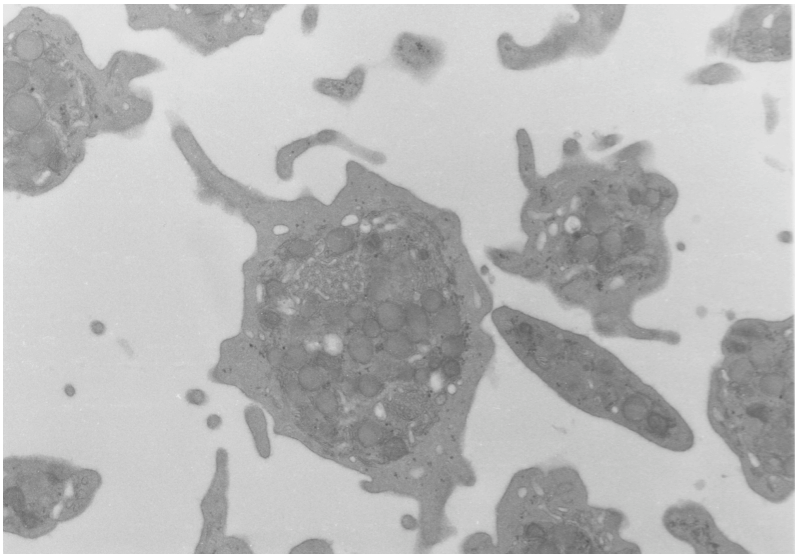


Figure 1.3 Representative TEM showing activated platelets
A transmission electro micrograph (TEM) of a representative population of activated (ET-1 and 5HT) platelets showing the spike-like surface extensions (pseudopodia), which is accompanied by surface enlargement. There is a rearrangemets of the microtubules which are translocated centripetally.

Initially, when the platelet contacts an injured surface it looses its disc shape and adapts a more rounded one. Secondly, finger-like projections (pseudopodia) grow from the cells periphery as the platelet flattens over surfaces and broad lamellae are extended (**Jagroop *et al.,* 2000).** On flattening the platelet granules and organelles become

concentrated into the centre of the cell, resulting in a 'fried egg' appearance. Finally, a dynamic phase of membrane motility begins along various points along the lamellae (membrane ruffles form and retract inward) (see figure 1.3). During the platelet activation, the actin filaments contents doubles from resting platelet concentration of 0.22 to 0.44 mM. As mentioned earlier, the first change to occur upon activation is when the normal disk shape platelet converts into a compact sphere with long dendritic extensions to facilitate adhesion (Grundmann *et al.,* 2003).

The conversion of resting disc shaped platelet into a rounded shape occurs if the cytoplasmic calcium levels rise into the micromolar range (Hayward *et al.,* 2006). The resting platelet maintains a cytosolic calcium of 10 to 20 nM (Harrison *et al.,* 1999). Intracellular calcium may increase to near 10 μM when calcium channels open after activation of the phospholipase C pathway. That is when the enzyme hydrolyses polyphosphoinositide ($PI_{4,5}P_2$) forming a second messenger diacylglycerol and inositoltriphosphate (IP_3) (Knofler *et al.,* 1998). IP_3 is soluble, thus it diffuses into the cytoplasm moving to the receptors on the dense membrane of the platelet, where it binds and releases the stored calcium (Escolar *et al.,* 1999). Phospholipase C is activated by the βγ-subunit of trimeric G-proteins that couples it to serpentine receptors. These include protease-activated receptors, the ADP receptors ($P2Y_1$ and $P2Y_{12}$) and the 5HT receptor.

PSC is driven by rapid remodeling of the platelet cytoskeleton that is dependent on the new assembly of actin filaments (Jilma, 2001). This process is a complex one that is outlined below. The protein activated by calcium to remodel the cytoskeleton is gelsolin, an 80-kDa protein. Gelsolin contains two main actin-binding sites that are activated by

increased calcium levels. In the initial stage of cytoskeletal remodeling, the long filaments that exist in the resting cell are converted to many short filaments when platelets change from a disk shape to rounded. This process is mediated by the actin-severing property of gelsolin as calcium levels increase. In order to allow platelet spreading the rounded platelet must assemble new filaments and does this at the cell cortex by elongating the barbed end of the short filament fragments and by generating new barbed ends for monomer addition.

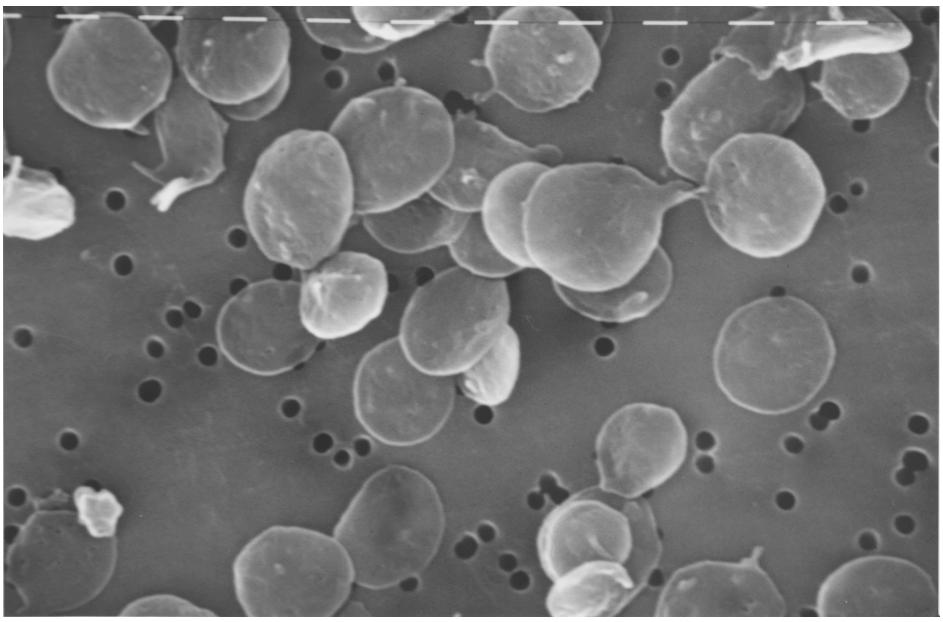


Figure 1.4 Representative ScEM showing resting platelets
Scanning electron microspy (ScEM) showing a representative population of normal resting platelets. Platelets appear to be disc shaped, and are 'separated' from each other.

The resting platelet stores actin in a monomeric complex with β4-thymosin and profiling. The ends of the actin filament have different affinities for actin monomers, with barbed ends having a 10-fold affinity for the monomer. Thus, the polymerisation reaction of

the barbed end of the growing filament provides a force to push out the finger-like filopodia and lamellipodia. A complex protein called Arp 2/3 is activated to generate new barbed ends when gelsolin and other proteins that cap the barbed ends of the actin filaments are removed (Cohen, 1979; Zucker *et al.,* 1985). The arrangement of the actin filament establishes the shape of the protrusion and polymerisation of actin filaments. Filopodia are tight bundles of actin filaments that originate near the centre of the platelet (Cohen, 1979; Cohen *et al.,* 1982). Figure 1.3 of resting platelets as seen with ScEM is shown above.

The next stage as mentioned above is when the platelet flattens over surfaces and broad lamellae extend (Boyles *et al.,* 1985; Zucker-Franklin, 1970). The lamellipodia of the spread platelet are organised into a three dimensional network of cross-linked actin filaments (see figure 1.5). The considerable re-organisation of the actin cytoskeleton is also accompanied by the exocytosis of the platelet storage granules (Boyles *et al.,* 1985; Zucker-Franklin, 1970). The stage of platelet activation that follows PSC is platelet aggregation (described below in section *1.4.3*)

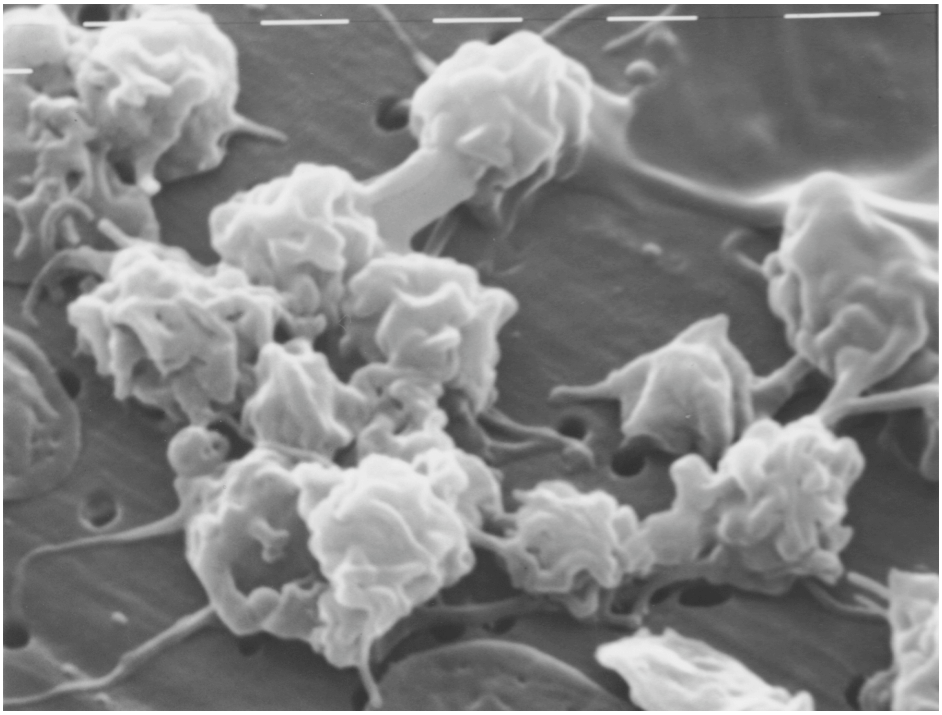


Figure 1.5 Representative ScEM showing activated platelets
Scanning electron microscopy (ScEM) showing a representative population of activated (with ET-1 and 5HT) platelets. There is a more rounded shape with finger-like projections (pseudopodia) which grow out from the cells periphery.

1.3.3 Platelet Receptors

Since platelets lack a nucleus, they cannot adapt to different situations by *de novo* protein synthesis. Thus, in order to compensate and deal with physiological functions, platelets possess several mediators in their storage granules (Cramer, 1999; Italiano, Jr. *et al.,* 2003). Because of the relatively small size of platelets, their membrane and membrane proteins represent a larger proportion of cellular mass compared with other cells. Moreover, since the main function of platelets is haemostasis, it is not surprising that their major receptors have a direct role in this process, either in platelet activation, or as adhesive

receptors interacting with damaged cell walls or with other platelets that contribute to a thrombus formation (Ishii, 2011)

As well as being involved in haemostasis platelets are involved in a range of less well understood functions like inflammation, anti-microbial host defense, tumour metastasis and angiogenesis. Thus, platelets express a range or receptors with no obvious direct role in hemostasis, but they could be implicated in other activities like immunological defense against viruses, bacteria, and parasites among other pathogens. Many of the receptors are present in low copy numbers, however they act synergistically with major agonists and have critical roles in regulating overall platelet responsiveness, thus they are physiologically important. With present technology, we have identified a number of platelet receptors and their demonstrable physiological function detected on platelets (see table 1.2).

Table 1.2: Platelet Plasma Membrane Receptors

Name	Category	Ligand	Effect
GPIIb/IIIa	Integrin	Fibrinogen vWF	PLT aggregation
GPIb/IX/V	LLR	vWF Thrombin	PLT adhesion
PAR 1 PAR 2 $P2Y_1$ $P2Y_{12}$	7-TMR	Thrombin ADP	PLT activation: Aggregation & PSC (**Jagroop** *et al.,* 2003a)
GP VI	Ig Gene	Collagen	PLT activation:
P-selectin	Lectin	PSGL	WBC tethering & binding

1.4 Laboratory Investigation of Platelets

Several laboratory techniques have been used to assess platelet function. In this thesis only some of the many techniques currently available are mentioned. Each test has its advantages and disadvantages and the therefore, results should be interpreted in the context of patient history and examination. The ideal test should be quick, simple, inexpensive, reproduceable and non-invasive (Harrison, 2000). Currently available tests can measure platelet number, size, their capacity to aggregate, and the concentration of release substances or the expressed receptors of activated platelets. However, accurate assessment of platelet count and function is highly dependent on sample collection and preparation (Barradas *et al.,* 1993). Selection of subject/patient is the first factor to be considered. Blood donors should be relaxed and not have taken any anti-platelet agents for at least 1 week (preferably 2 weeks) preceding blood sampling (Harrison, 2005). In order to reduce stasis, it is recommended that the blood is collected using 19-21 gauge needle, and with plastic syringes, and which also prevents platelets from sticking to the sides (Harrison, 2005). In addition, the first aliquot of the sample should be discarded.

1.4.1 Choice of Anti-coagulant

An important factor to consider when collecting blood is which anti-coagulant to use as this affects both platelet count and functionality. The most recommended (depending on the test) is trisodium citrate (0.102 or 0.129 M, buffered or non-buffered) at a concentration of 9 parts blood to 1 part trisodium citrate solution. This anti-coagulant causes a lowering of

ionised calcium thus preventing coagulation (see Appendix A, PSC & Appendix B, platelet aggregation references authored by **Jagroop *et al.)***

Heparin inhibits the generation and activity of thrombin via its complex with anti-thrombin III. Heparin may be considered unsatisfactory if a centrifugation step is required (Mikhailidis *et al.,* 2002). This is because of platelet sedimentation with red cells. Thus, in platelet rich plasma (PRP) the platelet count may be significantly lower when collected in heparin as compared with citrate (**Jagroop** *et al.,* 1996; **Jagroop** *et al.,* 1999; Mikhailidis *et al.,* 1996;Mikhailidis *et al.,* 1986; Mikhailidis *et al.,* 1987). In addition, platelets may aggregate or adhere to the sides of the containers giving a false low platelet count in PRP.

The use of ethylene-diamine-tetra-acetic-acid (EDTA), (Dastjerdi *et al.,* 2006) can lead to calcium chelation, and thus a lower platelet count as cells aggregate. Since platelet aggregation is dependent on the presence of calcium, EDTA is not suitable for use in platelet aggregation studies. Furthermore, EDTA expands platelet volume (**Jagroop** *et al.,* 2003b).

Hirudin, may be used as it preserves the concentration of ionised calcium necessary for certain tests, but this anti-coagulant is considered too expensive for routine tests (Danchin *et al.,* 2010; Rao *et al.,* 2010).

D-phenylalanine-proline-arginine chloromethyl ketone (PPACK) is an anti-thrombin that does not cause calcium chelation and therefore does not exert an effect on platelet function based on available calcium. This anti-coagulant may be useful when assessing the platelet inhibition by GPIIb-IIIa antagonists like eptifibatide, abciximab and tirofiban (Hobbach *et al.,* 2003; Proimos, 2001; Verstraete, 1995). However, PPACK is relatively

expensive and requires higher concentration in relation to other anti-coagulants to prevent platelet clotting (Verstraete, 1995).

Acid-citrate-dextrose (ACD) (Mackenzie *et al.,* 2010), an anti-coagulant, is useful for washing or gel-filtering platelets. It prevents platelet aggregate formation in the centrifugation process (Groh *et al.,* 1993). ACD alters the pH of PRP to 6.5 and is thus unsuitable for platelet aggregation experiments. However, ACD-A, a formulation of ACD is acceptable for platelet aggregation testing since it maintains the PRP at a pH of 7.2 (Cazenave *et al.,* 1983; Groh *et al.,* 1993).

1.4.2 Bleeding Time

One of the oldest methods (developed in the early 1900s) to assess platelet function is bleeding time (BT). Various methods have been used to assess BT. The Duke technique is where a small incision is made on the ear-lobe, and the Ratnoff technique is when an incision is made on the ball of the finger (Harrison, 2005). The bleeding time as designed by Duke was thereafter modified and known as the Ivy test (Harrison, 2005), which is a combined bleeding time-tourniquet test (Bock *et al.,* 1999). In fact, by 1984 a survey of current practice in the BT test was undertaken by the National External Quality Assessment Scheme (NEQAS), UK in blood coagulation. Most centers (88.5%) performed BT and of these, the Ivy test was the most commonly performed. Only 13.6% performed the Duke method. The use of a commercial template method, known as 'the Simplate', provided a measure of agreement among the hospitals using this instrument (Hayward *et al.,* 2006). In one study the authors compared the sensitivity and the reproducibility of the BT techniques

according to Ivy and Simplate II (Hayward *et al.,* 2006). They concluded that the Simplate II method is not superior in sensitivity or reproducibility to the Ivy method, which is cheaper, takes less time, and does not leave scars (Moake *et al.,* 1988). There was however, some contradiction regarding the BT test. In a study, Bowie et al. (Shenkman, 2003) concluded that BT when properly standardized, is an important test in the evaluation of haemostatic disorders. Moreover, the positive aspects of this test is that it does not require expensive equipment or even a laboratory and is not affected by the variables associated with blood sampling and anti-coagulation (Morrison, 2007).

However, it is now widely regarded that there are only four clinical reasons for using the BT test: (1) as a screening test for patients before invasive procedures, (2) to determine the cause of ongoing bleeding, (3) to explain previous episodes of bleeding, and, (4) to diagnose hereditary bleeding disorders e.g. vWD (Gobbi *et al.,* 2006). Nowadays assessing BT even for these reasons is considered not be as accurate as once thought. This was demonstrated when a MEDLINE search was done to assess the accuracy of BT as a screening test for patients; BT often failed to provide sufficient data for calculation of sensitivity and specificity of this test (Gibbs, 2009; Michelson, 2009). One study showed that 14 % patients who underwent cardiac surgery had excessive bleeding, but their BTs were not different from those of the remaining 86 % who did not bleed (Michelson, 2009). Furthermore, studies of the predictive value of BT in cardiac surgery and non-cardiac surgery demonstrated that BT does not provide conclusive evidence (Michelson, 2009; van Werkum *et al.,* 2008).

To date the disadvantages of BT far outweigh the advantages with the main one being the reproducibility of the method; it also appears to be a non-specific and insensitive test (van Werkum *et al.,* 2008). The skill of the technician greatly affects the results, thus there is a high interoperator variability (Barradas *et al.,* 1988). A lack of consistency can even be due to skin thickness and temperature (Accumetrics Inc., 2007). Frequent scarring of the subjects have also been reported (van Werkum *et al.,* 2008). Thus, BT is no longer recommended as a clinical test for platelet function, and has been discontinued in most centres.

1.4.3 Platelet Shape Change and Platelet Aggregation

The initial reaction of platelet adhesion to vessel endothelium is PSC, which is followed by platelet aggregation. PSC is an early phase of platelet activation that precedes platelet aggregation. Many techniques have been developed to assess platelet activation. The measurement of PSC is one such method. Turbidometric aggregometry (using PRP) is one of the several techniques used to assess PSC (Barradas *et al.,* 1992; Frojmovic *et al.,* 1982; O'Brien *et al.,* 1966). However, this is at best a semi-quanti-tative method; it is an estimation of the increase in the optical density in the phase preceding aggregation (Barradas *et al.,* 1992; Frojmovic *et al.,* 1982; O'Brien *et al.,* 1966). In this thesis, a high-resolution (0.07 fl) channelyzer (ZM34 coupled to a Coulter counter) was used to determine the apparent size of platelets. The sensitivity of this method can be attributed to the fact that this channelyzer uses 256 channels in comparison to those previously used by others, that had only 128 and therefore was a lower resolution (Frojmovic *et al.,* 1982; Gear, 1981; O'Brien *et al.,* 1966;

Sanderson *et al.,* 1996). Moreover, this technique is suited to assessing small increments in median platelet volume (MPV) that are associated with PSC induced by low doses of agonists (**Jagroop** *et al.,* 2000a; **Jagroop** *et al.,* 2001; **Jagroop** *et al.,* 2003a**; Jagroop** *et al.,* 2004; **Jagroop** *et al.,* 2008) (*see Appendix A: PSC reference list with **IA Jagroop**)*. It has previously been shown that PSC, which is accompanied by an increase in MPV, occurs concomitantly with morphological changes in human platelets (**Jagroop *et al.,*** 2000a).

Our previous studies support others who suggest that inducing an increase in median platelet volume (MPV) requires lower concentrations of agonists than those required to initiate aggregation (Sanderson *et al.,* 1996). The PSC phenomenon is essentially aspirin-resistant because arachadonic acid (AA) metabolism is not involved in this phenomenon except when this fatty acid is used to activate platelets (Barradas *et al.,* 1992; Siess *et al.,* 1984).

With the use of the channelyzer method, PSC in several situations of potential clinical relevance have been demonstrated, as described below. For example, thrombin-induced PSC is inhibited *in vivo* by the addition of either UH or low molecular weight heparin (LMWH, nadroparin) (Jagroop *et al.,* 1996; Siess *et al.,* 1984). In addition, doxazosin, an alpha 1-adrenoceptor antagonist, used in the treatment of essential hypertension and/or benign prostatic hyperplasia inhibited serotonin (5HT)-induced shape change in human platelets **(Jagroop** *et al.,* 2001; Siess *et al.,* 1984). In another study, we showed that naftidrofuryl (Praxiline) which is used to improve claudication in (peripheral arterial disease) PAD was able to reverse the PSC induced by endothelin-1 (**Jagroop** *et al.,* 2000d; Siess *et al.,* 1984). The effect of endothelin-A (ET-A), BQ123 and ET-B, BQ788

receptor antagonists on PSC induced by ET-1 was investigated. We hypothesized that both ET(A) and ET(B) receptors might contribute to the ET-1-induced PSC (**Jagroop** *et al.,* 2000d; Siess *et al.,* 1984). Losartan, a selective angiotensin II antagonist, is used in the treatment of hypertension (Chang *et al.,* 2010; Russell *et al.,* 2011). Using the PSC methodology, it was shown that losartan at concentrations similar to those achieved in the circulation during treatment, significantly inhibited angiotensin II- as well as U46619 (a thromboxane A_2 (TXA_2) analogue)-induced PSC. Thus, suggesting that losartan, in addition to its blood pressure lowering action, also appears to have anti-platelet activity (**Jagroop** *et al.,* 2000c; Siess *et al.,* 1984). Indeed, we demonstrated for the first time using human platelets as a neuronal model with our PSC method, that an appetite suppressant, dexfenfluramine (previously used to treat obesity) showed serotonergic action (**Jagroop** *et al.,* 2000b; Siess *et al.,* 1984). These drugs (dexfenfluramine and fenfluramine) were voluntarily withdrawn due to reports of adverse effects on heart valves (1997c; McCann *et al.,* 2007; Wee *et al.,* 1998).

The MPV (a measure of PSC) was also assessed in patients with PAD, where platelet hyperactivity has been reported (Barradas *et al.,* 1993; Barradas *et al.,* 1994b; Siess *et al.,* 1984). Patients with PAD have elevated plasma levels of 5HT and reduced intra-platelet levels of this bioamine (Barradas *et al.,* 1994a;Cheshire *et al.,* 1996; Fawcett *et al.,* 1998). Other bioactive compounds (e.g. ADP, and TXA_2) are also released upon platelet activation. It is therefore of potential clinical relevance that various platelet agonists induce PSC (Barradas *et al.,* 1994a; Cheshire *et al.,* 1996; Fawcett *et al.,* 1998). Thus, from some of our previous studies (outlined above), we have documented that selective antagonists block the

increase in MPV as induced by these various agonists. These observations can be considered as evidence that MPV measurements represent, specific, receptor-mediated, phenomenon.

This PSC method usually only requires low concentrations agonists, which is sufficient in PRP to induce a response in that allows antagonist inhibition. High agonist levels would not allow us to see these responses at pharmacological levels of antagonists. Also, it should be noted that when measuring PSC we are assessing inhibition in the early phase of platelet activation. With this in mind, in this thesis we showed that it was possible to establish that both ADP receptors $P2Y_1$ and $P2Y_{12}$ were involved in the PCS phenomenon in human platelets **(Jagroop** *et al. 2003a).*

Platelets interact with each other to form aggregates. Platelet aggregation can be quanti-fied by aggregometry. The original aggregometer which consisted of an absorbptiometer was first described by Born in 1962 followed by O'Brien, which became the de facto "gold standard". The most commonly used method nowadays is a spectrophotometer attached to a chart recorder. This requires the preparation of PRP, which is set in the spectrophotometer as the most opaque setting possible (0% aggregation) and the platelet poor plasma (PPP) is set as the most transparent situation possible (100% aggregation). In response to an agonist platelets aggregate and the sample becomes "more clear" and the increased light transmission (or infrared signal) through the test sample is recorded. Platelet aggregation is calculated by dividing the distance from baseline to aggregation achieved, by the distance from the baseline to the theoretical 100% aggregation (PPP). Platelet aggregation has obvious disadvantages, the main one being the absence of red cells and an obligatory centrifugation step for PRP preparation. For these reasons, in the

early 1980's new methodological methods for the study of platelet aggregation were developed.

The most common whole blood method for platelet aggregation is electrical impedance that measures the amount of activated platelets accumulating across electrodes. The electrical impedance method may also be used with PRP. Single platelet counting methods became popular among researchers (Fox, 1982). In this thesis, platelet aggregation was measure in whole blood with the use of an $A^{C}T$ diff Analyzer (Beckman Coulter, Inc, Fullerton, CA) (*see Appendix B, platelet aggregation reference list authored by **IA Jagroop**)*. An initial baseline value was obtained in anti-coagulated blood, this was regarded as 100%. Then platelet counts obtained after the addition of various agonists were calculated as a percentage of the free platelet count in reference to the 100% baseline value. The final calculation is considered as the percentage of platelet aggregation that occurred in the sample (more details of the methods for whole blood platelet aggregation are described in chapter 2, section 2.7).

This method still has some disadvantages even with the inclusion of red cells. For example, this method is not available at the bedside. The assay is time consuming and the blood sample is required to be sent promptly to an onsite laboratory for testing. Also, there is considerable interlaboratory variation when performing platelet aggregometry. This may be due to the training of the technician, selection of agonist concentration, the adjusted platelet concentration that is required as the standard in each assay, processing temperature, completion time of the assay, and the rate of stirring samples. With these limitations, there is difficulty in comparing the results between laboratories.

1.4.4 Platelet Function Analyzer-100®

Automated whole blood platelet function screening assays like the Platelet Function Analyzer-100 (PFA-100®) are gaining popularity even though they do not measure the vascular component of the BT (Steinhubl *et al.,* 2001). A study compared BT and PFA-100® as screening tests for platelet dysfunction in 113 hospital inpatients. The results demonstrated improved sensitivity of the PFA-100® over BT and whole blood platelet aggregation studies supported the PFA-100® results (Neri Serneri *et al.,* 2004). Thus, PFA-100® is now available as an alternative to BT (van Werkum *et al.,* 2008), with more that 500 publications to date (MEDLINE accessed 8th Feb 2011). This instrument provides a quantitative measure of primary, platelet-related haemostasis at high shear stress. The PFA-100® measures the fall in flow rate as platelets within citrated blood are aspirated through a capillary and begin to seal an aperture (150 μm) within a membrane. The membrane is treated with collagen and epinephrine (CEPI) or collagen and ADP (CADP). The final closure time (CT; time taken to occlude the aperture) and the volume of blood that has been aspirated through the aperture are recorded. Closure time can be measured to a maximum of 300s.

The PFA-100® has been used to provide rapid results as an early indication of a potential bleeding problem. Also, PFA-100® Closure time (CT) represents a fast, simple and sensitive method of assessing glycoprotein IIb/IIIa antagonism *in vivo*; it is comparable to optical aggregometry, and suitable for testing larger numbers of glycoprotein IIb/IIIa antagonists (Hayashi *et al.,* 1989; Kumlin *et al.,* 1986; Lawson *et al.,* 1986; Murphy *et al.,*

1994). However, PFA-100® may not be sensitive enough for monitoring clopidogrel therapy (Kumlin *et al.,* 1986).

PFA-100® has both good intra-assay and inter-laboratory reproducibility, with the sample error being reported at approximately 10% (Murphy *et al.,* 1994). However, there are a number of variables that may affect PFA-100® testing, for example, anti-coagulation concentration. (Fitzpatrick *et al.,* 1977). Another factor that affects PFA-100® is platelet number. That is to say, that when a haematocrit is 10% or less or a platelet count is $10x10^9$/L the test will result in non-closure The PFA-100® has many advantages as a screening test to measure platelet function. Firstly, it requires whole blood eliminating the need for sample preparation (Sakariassen *et al.,* 1983). In addition, the whole blood is collected in citrate, which therefore allows samples to be shared for other coagulation tests. The test requires very little blood (only 0.8 ml/cartridge). An attractive feature of the PFA-100® system is its simplicity and ease of use without the need for operators to have specialised knowledge or training. It produces remarkably rapid and reproducible results with good precision (Baumgartner *et al.,* 1972; Sakariassen *et al.,* 1983).

There are disadvantages associated with the PFA-100® system. Currently, only two types of cartridges are available (Sixma *et al.,* 1998). Also, even when a prolonged CT is obtained and a platelet disorder is indicated, results cannot distinguish severe platelet disorders from vWD and thus its specificity is limited (Sixma *et al.,* 1998). The test can also sometimes give false-positive results in patients who have normal platelet function (Roald *et al.,* 1994).

It is therefore recommended that when doing a PFA-100® test, a full blood count should always be performed to exclude thrombocytopenia or anaemia (Kaplan *et al.,* 1981). Also PFA-100® should be used as a screening tool and alongside already existing platelet function tests (Wohl, 1981).

1.4.5 Impact Cone and Plate(let) Analyzer

The Impact (DiaMed, Cressier, Switzerland) was designed to test platelets under close to physiological conditions. Circulating platelets interact with and adhere to the exposed sub-endothelial extracellular matrix under flow, thereby forming platelet aggregates leading to bleeding arrest. It is therefore essential to study platelet function under the conditions that contribute to the physiological parameters that play a role in platelet adhesion and aggregation. The physiological milieu of platelets, includes flow, red blood cells and other blood components. This currently available technique uses a polystyrene surface on which plasma proteins, namely fibrinogen and vWF (Reikvam *et al.,* 2009), are immobilised and form a thrombogenic surface. One of the advantages of the method is that it only requires a small volume of whole blood (130 -200 μL) anti-coagulated with tri-sodium citrate (3.8%) this is placed on the plastic and a defined shear rate of 1800 sec^{-1} is applied using a cone and plate device. The next stage involves staining and measuring the percentage of surface covered (SC) by the stained objects and measuring the average size of the adherent particles using and image analyser. Results show that under these conditions only platelets and not other blood cells will adhere to the surface and forms elongated aggregates that align with the laminar flow lines. With normal blood, platelet deposition is a

shear- and time- dependent process that reaches a maximum level within 2 min at high shear rate (1800 sec^{-1}).

CPA has been useful in the assessment of platelet function in a variety of normal and clinical situations. For example, in full-term and pre-term babies (Luddington, 2005), in patients with diabetes (Luddington, 2005), thrombocytopenia (Luddington, 2005; Reikvam *et al.,* 2009), vWD (White, 1998), thrombotic thrombocytopenic purpura (Clauser *et al.,* 2009), phospholipid syndrome (White, 1998) and for testing platelet function in stored platelet concentrates (Clauser *et al.,* 2009; Nurden, 2005). Platelet aggregation was assessed by light transmission aggregometry (Dager *et al.,* 2004) and Impact-R.

The Impact is entirely dependent on the functional state of the GPIIb-IIIa and the GPIb-IX receptors and their ligands (fibrinogen and vWF) making it a useful tool for testing the response to GPIIb-IIIa antagonists (Jaumdally *et al.,* 2007).

In summary, the Impact cone and plate(let) analyser is based on the principle of quanti-fication of high shear platelet adhesion/aggregation onto a surface (Threatte, 1993). It has been used in the detection of inherited and acquired defects in primary hemostasis (Blann *et al.,* 2003; Lim *et al.,* 2004), detection of platelet hyperfunction and monitoring anti-platelet therapy (Leung *et al.,* 1993; Treasure *et al.,* 1995). To date, the main disadvantage of this technique is that there is little widespread experience. However, the use of the Impact cone and plate analyser may gain popularity because of its advantages which include: (a) the use of a small volume of blood, (b) the technique is rapid and simple, and, (c) this unique method gives both visual and numeric evaluation of both platelet adhesion and aggregation properties under close to physiological conditions.

1.4.6 The VerifyNow System

The VerifyNow system has the advantage over platelet aggregation because it is one of the most user-friendly point-of-care platelet function tests and it produces rapid results. However, although this device is simple and rapidly performed, it is not as sensitive as the laboratory platelet aggregometry (Patrono *et al.,* 2004b). The machine has the same fundamental advantages as platelet aggregometry in that it measures the most important function of platelets, which is their ability to aggregate in a GPIIb/IIIa-dependent manner (Maron *et al.,* 2000). The VerifyNow system measures light absorbance. Citrated blood is inserted into the single-use cartridge that contains biochemical reagents, agonists and fibrinogen coated beads. Depending on the inhibitory level of the anti-platelet drug, specific agonists activate the platelets. As a result, the activated platelets cause agglutination and fall out of solution. It is this rate and extent of platelet-induced agglutination that is measured (1996 *no authors listed*). Verify Now previously known as the Ultegra Rapid Platelet Function Analyzer currently measures three types assays; the IIb/IIIa assay (sensitive to GPIIb/IIIa antagonists, like abciximab), the aspirin assay (sensitive to aspirin), and the $P2Y_{12}$ receptor assay (sensitive to thienopyridines) (Farhan *et al.,* 2010; Pfisterer *et al.,* 2010).

The first type of assay using the GPIIb/IIIa cartridge contains fibrinogen coated polystyrene beads and thrombin receptor activating peptide (1999 *no authors listed*; Lagerqvist *et al.,* 2005) as agonist (1996 *no authors listed*). The second VerifyNow assay specifically monitors the inhibitory effect of aspirin therapy. The agonist in this cartridge is AA in a final concentration of 1mmol/l. AA is converted by the COX enzyme to TXA_2 (Neri

Serneri *et al.,* 2004; 1996 *no authors listed*; 1997b *no authors listed*). This assay can be used to detect aspirin responders from non-responders (Casella *et al.,* 2003). The third VerifyNow assay is to monitor the inhibitory effects of thienopyridine therapy, the $P2Y_{12}$ receptor assay. The cartridges for this assay contain two different chambers for agonists. Chamber 1 contains thrombin receptor activation peptide (TRAP) which determines a maximal baseline of platelet function measure and chamber two contains ADP (20 μmol/l) and PGE_1 22 nmol/l.

The VerifyNow $P2Y_{12}$ receptor assay is more sensitive for the specific ADP-$P2Y_{12}$ receptor pathway since PGE_1 is added to suppress platelet activation, which contributes from ADP-binding to the $P2Y_1$-receptor (1997b; Diehm *et al.,* 2004). Results from this assay are reported as $P2Y_{12}$ receptor reaction units percentage inhibition and base. The VerifyNow $P2Y_{12}$ receptor assay has been used to evaluate the anti-platelet effects of clopidogrel (Ahn *et al.,* 2011; Malinin *et al.,* 2006).

Although this technique had the advantage over other techniques for its use in point-of-care testing, the disadvantage remains that it is one of the most expensive platelet function assays (Michelson *et al.,* 2006; Michelson, 2004).

1.4.7 Flow Cytometry

Flow cytometry has been used to identify activated platelets in patients with unstable angina, stable CHD, pre-eclampsia, cerebrovascular ischemia, acute MI, and coronary angioplasty (1997b *no authors listed*; Patrono, 1994). Since platelets undergo changes during storage, flow cytometry is useful to monitor the quality of blood bank platelet

concentrates (Ehrman *et al.,* 1980). Platelet membrane GP deficiencies are also evident with flow cytometry. Thus, major platelet disorders like Glanzmann thrombasthenia or Bernard Soulier syndrome can be diagnosed with the appropriate monoclonal anti-bodies (Mylotte *et al.,* 2011). Other uses of flow cytometry include monitoring anti-platelet agents (e.g. thienopyridines, GPIIb/IIIa antagonists and aspirin), and to evaluate patients with immune thrombocytopenia and alloimmunisation (Scharf *et al.,* 2011).

Platelets are labeled with a specific anti-body conjugated to a fluorescent probe such as fluorescein isothiocyanate, peridinin chlorophyllprotein or phycoerythrin. In the flow cytometer the cell suspension which includes labeled platelets passes through a flow chamber and across a focused laser beam (Gurney *et al.,* 2002; Lacroix *et al.,* 2010). The laser beam has a wavelength similar to that needed to excite the fluorescent molecule. The light emitted by each type of fluorescent molecule has a characteristic wavelength and is detected by the flow cytometer (Lacroix *et al.,* 2010; Siljander, 2011).

The obvious advantage of flow cytometry is that it can be used for a variety of tests. A small volume of whole blood is used, thus platelets can be directly analyzed in their physiological milieu including red and white blood cells (Mylotte *et al.,* 2011; Tarrant, 2005). Moreover, since whole blood is used minimum sample manipulation is required thus preventing the artifactual *in vivo* activation and potential loss of platelet sub-population. With flow cytometry both the activation state and the reactivity state of circulating platelets can be observed. Also, this method does not involve the use of any radioactive materials (Shafi, 1986).

Among the disadvantages of flow cytometry, are the high running cost of the equipment and reagents. Also, the preparation of the sample is complex and must be processed quickly. Analysis using this technique requires a highly trained technician. Flow cytometry allows us to analyze only the markers on circulating platelets. Thus, if the platelets are activated and rapidly cleared they may not be detected (Gurney *et al.,* 2002).

1.4.8. Thromboxane A_2 Generation

Thromboxane A_2 (TXA_2) is synthesised by activated platelets and its release signals that further stimulate platelet activation at the site of injury where additional platelet recruitment forms a clot. TXA_2 a potent vasoconstrictor can stimulate mitogenesis and accelerates haemostasis and proliferation at vascular injury (Bhavaraju *et al.,* 2010; Moliterno, 2008). The biosynthesis of TXA_2 is increased in clinical syndromes where platelet activation is prevalent, for example, myocardial infarction, severe PAD, preeclampasia, scleroderma, sickle cell anaemia, pulmonary hypertension and stroke, to name a few (Drouet *et al.,* 2010).

TXA_2 is rapidly converted to the more stable and inert compound TXB_2. This is further metabolised to 11-dehydro TXB_2. It is TXB_2 that is assessed to give an indirect measure of TXA_2 (Miura *et al.,* 1999; Seidel *et al.,* 2011). TXB_2 can be measured from clotted blood (at 37^0C) or from supernatants derived from PRP or purified platelets where cyclooxygenase COX-1 activity has been stimulated by agonists (FitzGerald *et al.,* 1987; Patrono *et al.,* 1990). The metabolite 11-dehydro TXB_2 can also be measured in urine by a commercially available assay (AspirinWorks®). The advantage of measuring serum TXB_2 is

that it is directly dependent on aspirin's target COX-1. The disadvantage of measuring serum TXB_2 is that it is an indirect measure in the sense that platelets are not directly assayed, and it may not be entirely platelet specific (Roth *et al.,* 1975a). Also, the measurement of serum TXB_2 or urinary 11-dehydro TXB_2 are not rapidly available to clinicians as point-of-care tests (Miura *et al.,* 1999; Seidel *et al.,* 2011).

Both quantitative immunoassays and standardised stable isotope dilution mass spectrometry have been the development to measure the two major metabolites of TXB_2 (Ehrman *et al.,* 1980). Kumlin and Granstrom developed a radioimmunoassay (RIA) for 11-dehydro TXB_2. They initially examined the chemical stability as well as its chromatographic properties in order to reliably assay 11-dehydro TXB_2. Two different anti-plasmas were raised in rabbits against conjugates of 11-dehydro TXB_2 with bovine serum albumin. These displayed somewhat different properties in their recognition of the two forms of 11-dehydro TXB_2. Thus, a radioimmunoassay employing these anti-bodies was developed (Roth *et al.,* 1975b). Recent instrumentation has become sufficiently sensitive to assay the TXB_2 metabolites by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), reducing sample preparation to solid phase extraction (1997b;Patrono, 1994).

The quantitation of TXB_2 by immunoassays or mass spectrometry (Loll *et al.,* 1995) have been useful in defining aspirin's dose-dependent inhibition of the capacity of platelets to form TXA_2 (1997b *no authors listed*; Patrono, 1994) as well as to validate the use of low doses of aspirin in cardiovascular prophylaxis (1997b *no authors listed*; Patrono, 1994).

1.4.9 Perfusion Chambers

In the early 1970's Baumgartner and Haudenschild introduced the first annular perfusion chamber (Aster *et al.,* 2004). It was then possible to study the influence of plasma components, platelet membrane glycoproteins, red blood cells and the shear rate of the blood on the platelet adhesion to the sub-endothelium of blood vessels (Weil *et al.,* 1995). Thus, for the past 35 years it has been recognised that shear forces generated by flowing blood have a significant impact on platelet adhesion and thrombus formation. Moreover, it became clear that studies on platelet adhesion and thrombus formation were only relevant when performed under conditions of flow (Liberopoulos *et al.,* 2006). This lead to the development of other types of flow chambers, including the flat perfusion chamber, where Sakariassen *et al* also made it possible to study isolated purified subendothelial proteins and isolated cultured vessel wall cells (Patrono *et al.,* 2004a).

Perfusion studies with human blood were traditionally performed in relatively large chambers requiring large volumes of blood, 55 ml circulated per min to achieve a typical shear rate of 1600 sec^{-1} (Farrell *et al.,* 1991). To address the problem of the need for large blood volumes, a newer perfusion chamber was developed with smaller dimensions. In comparison this used only 0.3 ml blood circulated per min for a shear rate of 1600/sec (Peters *et al.,* 2003). Having a smaller chamber has the advantage of a reduction in platelet activation occurring during perfusion. Also, less volumes of anti-bodies or inhibitors are required and it is possible to perform several perfusions with native blood from the same patient (Patrono *et al.,* 2004a). However, there are still some problems that need to be addressed, namely if a surface of laminin or thrombosponding is used, an uneven

distribution of adhered platelets is obtained. In addition, there appears to be sedimentation of red blood cells in the tubing when the flow rate is slow. Further research into the development of this type flow chamber was required.

Another problem faced by flow chambers was the presence of anti-coagulant when thrombus formation was studied. To overcome this problem an *ex vivo* perfusion system was developed which can be used with non-anti-coagulated blood (Diener *et al.,* 2004). The blood can be directly drawn from the antecubital vein through the perfusion chamber to better study the role of anti-thrombotic drugs (2002 *no authors listed*).

1.4.10 Additional Methods for Platelet Testing

In addition to the methods listed above, a few other tests can be used to assess platelet function. A summary of these platelet function tests is outlined below. The advantages, limitations and suitability for clinical applications are also mentioned.

The measurement of soluble platelet markers that are specific to platelet release are a means for detecting increased platelet activation *in vivo.* Platelet factor-4 (PF-4) and beta-thromboglobulin (*β*-TG) are stored in platelet α-granules and are secreted upon platelet activation (1990). These two proteins are specific to platelets that can be detected in both the MK and the platelet with the use of immunofluorescence and immunoperoxide methods (Juul-Moller *et al.,* 1992). Other soluble platelet release markers like sCD40L, sCD62P and GPV are usually detected by ELISA (1991 *no authors listed)*. However, the disadvantage of these methods is that they are prone to artefactual activation during blood collection and processing (Taylor *et al.,* 1999).

Thromboelastography (TEG) was originally developed more than 50 years ago (Farrell *et al.,* 1991). TEG records the continuous profiles of whole blood coagulation by measurement of the viscoelastic changes associated with fibrin polymerisation, and thereby provides a global assessment of haemostatic function (Yusuf *et al.,* 2001). The rate of fibrin polymerisation as well as the overall clot strength is assessed. The TEG or thromboelastometry (ROTEM) works on the principle where blood is incubated 37^0C in a heated cup where a pin is suspended and connected to a detector system. In the case of TEG it is a torsion wire and in the case of ROTEM it is an optical detector. The cup and pin oscillate relative to each other. As fibrin forms between the cup and the pin the transmitted rotation is detected and a trace is generated (Mehta *et al.,* 2001). TEG is a registered trademark of the Haemoscope Corporation. However, an alternative instrument marketed by Pentapharm GmbH uses an adaptation of the TEG, the rotational TEG or ROTEM (ROTEG). Thus, the term TEG/ROTEM is sometimes used. The main uses of the TEG/ROTEM have been to monitor blood component therapy during surgery, in the field of liver transplantation and it has also been useful in cardiac surgery (Mehta *et al.,* 2001). This instrumentation can also be used within surgical and anaesthesiology departments as point-of-care testing and for determining the risk of bleeding and as a guide to transfusion requirements. Thus, the main advantage of the TEG/ROTEM is that it is an inexpensive near-patient method for the quick evaluation of the patient's global haemostatic system (Steinhubl *et al.,* 2002).

Another method used in the diagnosis of platelet disorders is electron microscopy (EM) (Sabatine *et al.,* 2005). Since the development of EM, there has been a tremendous

insight into platelet structure, function, and their relation to pathology. This technique has helped to understand, characterize and classify many platelet disorders (1996 *no authors listed*). There are several instances where EM has been critical for identifying and characterising genetic problems. For example, platelet storage pool deficiency where there is less of the organelles that store adenine nucleotides, 5HT and calcium, which destined to be secreted during the platelet release reaction. EM has made it possible to distinguish various types of giant platelet disorders into distinct conditions based on the nature of platelet structural defects (Chen *et al.,* 2005). Thus, with the use of EM it has been possible to understand and classify platelet disorders that originate from platelet organelles, cytoskeleton and membrane defects. For example, grey platelet syndrome, Paris-Trousseau syndrome, MYH9-related thrombocytopenia's as well as Wiskott-Aldrich syndrome (Diener *et al.,* 2004). We have previously demonstrated that there is a relationship between ScEM and MPV in assessing PSC. An increase in MPV occurs concomitantly with morphological changes where the normally discoid circulating platelets become spherical and throw out from their surface, projections of varying shapes (**Jagroop** *et al.,* 2000a). This then develops into multiple pseudopodia resulting in a increase in the surface area (Diener *et al.,* 2004)**.** The main disadvantage of ScEM however, is that it requires expensive equipment, also it is only available in specialised units (Markus *et al.,* 2005).

Clinicians can utilize a range of tests for platelet function (some of which are outlined in this thesis), for the diagnosis and management of patients presenting with bleeding problems, atherothrombosis, to monitor the efficacy of anti-platelet drugs and to identify patients at risk of arterial disease. New platelet function tests are increasingly being

developed that aim to be simpler point-of-care instruments that can be useful pre-surgery/peri-operatively to aid in the prediction of bleeding and for monitoring haemostasis.

1.5 The Role of Platelets in Atherosclerotic Disease

PAD is defined as atherosclerotic occlusive disease of the extremities. PAD patients have are at a high risk of stroke, myocardial infarction (MI). Patients with PAD tend to have activated platelets (Bowbrick, 2003; Busti, 2010; Barradas, 1994; Barradas, 1993). This was supported by the findings of the Anti-platelet Trialists Collaboration (Drouet *et al.,* 2010), which showed that anti-platelet therapy reduces the odds of graft or native arterial occlusion in patients with PAD undergoing bypass surgery or angioplasty. Furthermore, a meta-analysis of anti-platelet agents in patients with PAD showed a benefit in terms of vascular events (Robless *et al.,* 2001). Platelets also play a major role in atherosclerosis and its complications in the coronary and cerebral vascular systems.

Platelets play a role in the development and progression of atherosclerosis. Injury to the arterial endothelial cell results in the endothelial cell dysfunction, the first step in the process of atherosclerosis. This injury causes a platelet response (see figure 1.6).

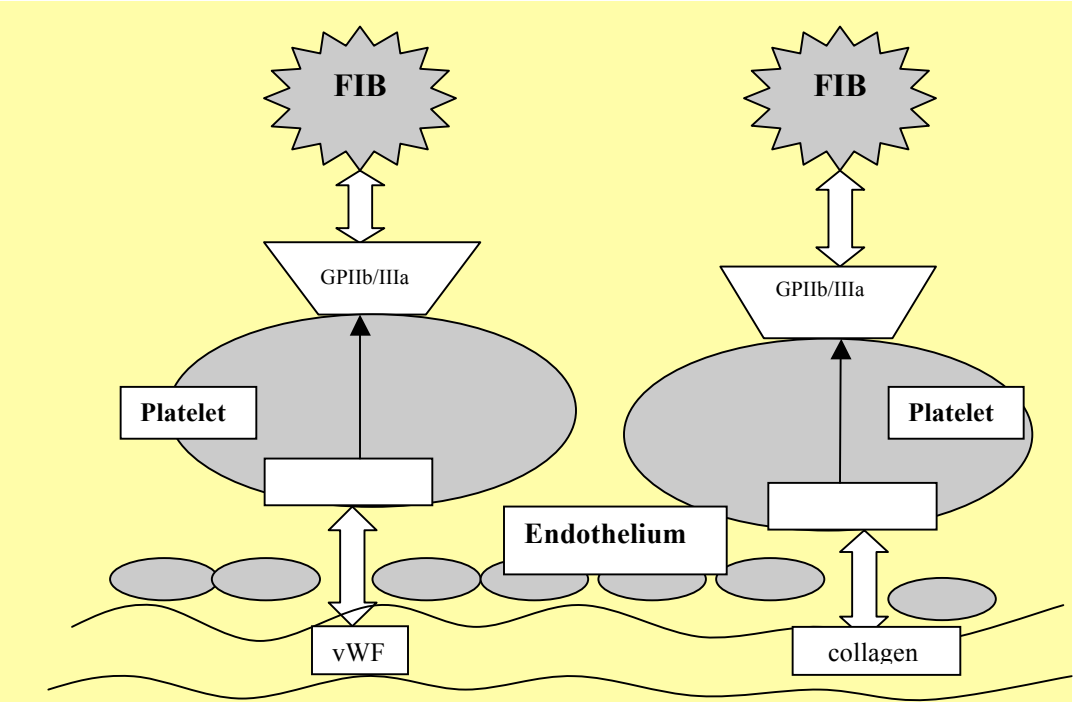


Figure 1.6: Activation and adhesion of platelets. Platelets adhere to the damaged endothelium in the presence of its components collagen and von Willebran factor (Moake *et al.,* 1988). Collagen interacts with glycoprotein Ia/IIa (GPIa/IIa) and vWF with GPIb/IX complex on platelets which results in platelet adhesion to the subendothelial matrix. The binding activates GPIIb/IIIa receptor within the platelet membrane, which changes its shape on activation to express a high-affinity binding site for fibrinogen (FIB). This process is called 'outside in' signalling and leads to further platelet activation, aggregation and accumulation at the site of injury.

Platelet adhesion leads to activation and the platelet degranulation of the platelet dense granules and the release of its contents like PDGF, ADP, α-granule proteins and β-TG. P-selectin which is present on the membranes of the α-granules of resting platelets will move to the outer membrane once activation occurs. A cascade of events involves the adhesion on monocytes on the luminal surface, which is then converted to activated

macrophages that take up lipoprotein particles to become foam cells. This leads to the formation of fatty streaks that contribute to the early stages of atherosclerosis.

The pathogenesis of atherosclerosis also involves the proliferation of smooth muscle cells. PDGF is important in this process and is essential for the migration of smooth muscle cells to the intima. PDGF receptors are found on the surface of smooth muscle cells, which upon stimulation by exogenous PDGF, results in smooth muscle cell proliferation, and synthesis of a PDGF-like protein that promotes proliferation of smooth muscle cell by stimulation of mitosis.

Studies have shown that the platelets of diabetic patients, tend to be hypersensitive to aggregating agents (Winocour, 1994). It was also observed that the release of α-granule content in vivo; activity of the AA pathway and prostaglandin and TXA_2 formation. Increased were increased (Winocour, 1994).

The increased platelet activity from diabetic patients parallels the enhanced vascular disease in diabetics (Winocour, 1994). In type 1 and 2 diabetes there is an association with impaired endothelial function. The decreased bioavailability of nitric oxide (NO), decreased production of prostacyclin and the increased production of TXA_2, affects the endothelium-dependent vasodilator function in diabetes. The decreased levels of NO associated with type 2 diabetes leads to increased platelet activation and aggregation and a greater risk in developing thrombosis and atherosclerosis (Nenci, 2000)

That is to say, the increased platelet aggregability and adhesiveness are due to the following (Winocour, 1994;Sobol, 2000;Nenci, 2000):

- A reduction in membrane fluidity
- Altered Ca^{2+} and Mg^{2+} homeostasis (increased intra-cellular Ca^{+} mobilization and decreased intra-cellular Mg^{2+})
- Increased AA metabolism
- Increased TXA_2 synthesis
- Decreased prostacyclin production
- Decreased NO production
- Decreased anti-oxidant levels
- Increased expression of activation dependent adhesion molecules (e.g. GpIIb-IIIa, P-selectin).

In type 1 and type 2 diabetes, platelets show enhanced aggregation at an early stage in the disease which precedes the development of CVD (Natarajan, 2008). Platelet hyperactivity may be due to a range of biochemical abnormalities, which include reduced membrane fluidity that can reflect changes in the lipid composition of the platelet membrane or even the glycation of membrane proteins (Natarajan, 2008).

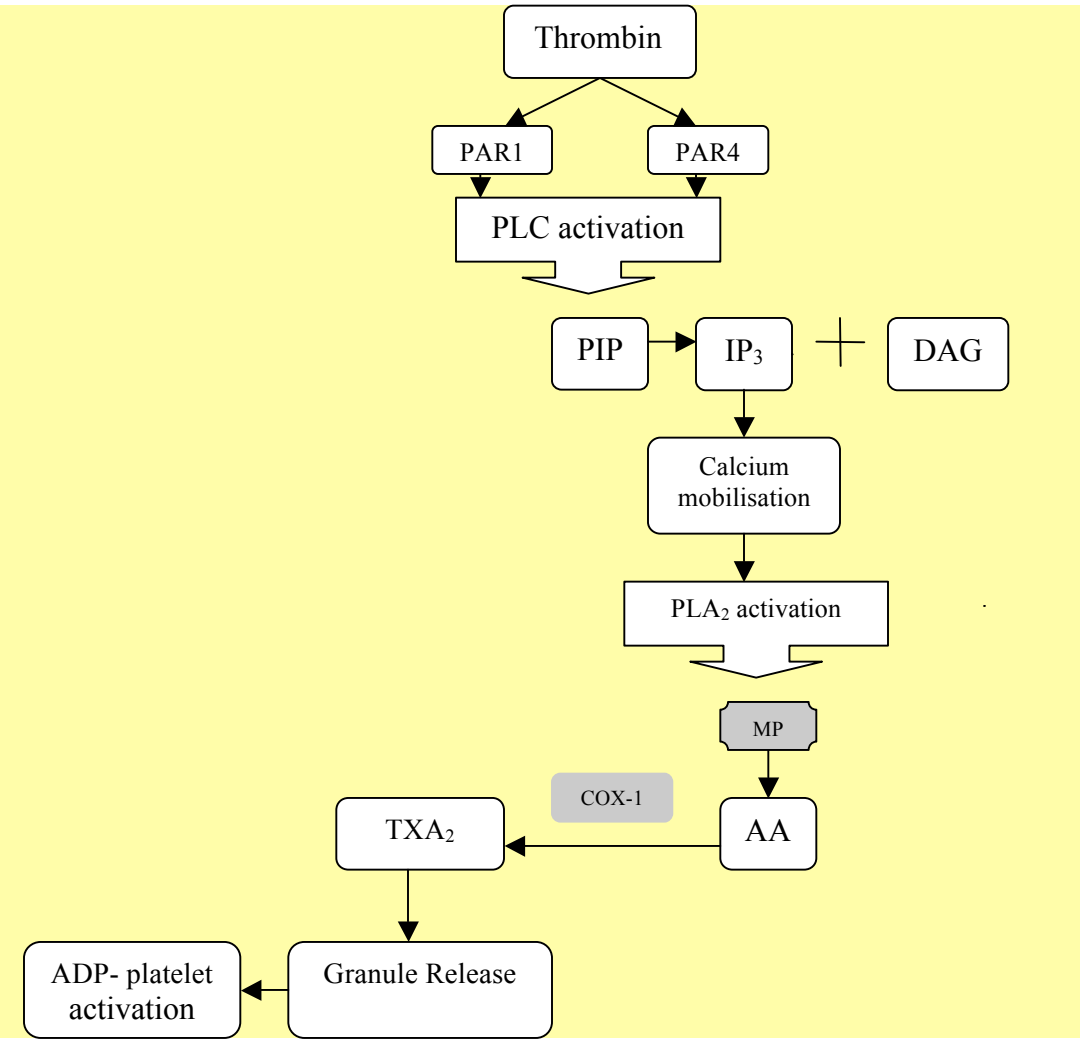


Figure 1.7: Platelet activation of the coagulation cascade via the IP_3 pathway.
Thrombin is generated after endothelial cell disruption and binds to protease-activated receptors 1 (PAR1) and PAR4. This activates phospholipase C and hydrolyses phosphatidylinositol 4,5 biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (Markus *et al.,* 2005). IP_3 mobilises calcium stores which activates phospholipase A_2 leading to arachidonic acid (AA) production. Cyclooxygenase-1 (COX-1) catalyses the synthesis of thromboxane A_2 (TXA_2) from AA in platelets, which leads platelet granule release, and further ADP-induced activation and aggregation.

The platelets from diabetic patients may also show increased AA metabolism, leading to enhanced TXA_2 production and enhanced platelet sensitivity. Calcium mobilization is increased from their storage pools and results in a higher concentration of intra-cellular calcium levels. This has been correlated with a reduction in membrane fluidity. Platelet hyperaggregability and adherence in diabetics is also associated with a reduction in intra-cellular Mg^{2+} (Vinik, 2001). Decreased NO and prostacyclin production in diabetic patients, results in the inhibition of platelet-endothelium interactions which leads to the endothelium-mediated vasodilation. In diabetic patients, increased aggregability and low vitamin C may be associated with a reduced anti-oxidant level (Carr, 2000)

Diabetics tend to have a larger number of platelets that express activation-dependent adhesive molecules like GPIIb-IIIa, P-selectin, lysosomal Gp53 and thrombospondin. Patients with diabetes have been shown to have increased levels of fibrinogen, which is consistent with elevated of platelet GPIIb-IIIa expression.

Finally, in patients with diabetes, the leading cause of disability and premature death is CVD. A patient with diabetes has a two-fold to four-fold increased risk of CHD, stroke and PAD. Both atherosclerosis and thrombosis contribute significantly to the increased risk of CVD in diabetic patients. Many studies have shown that anti-platelet therapy can be used in the treatment of diabetic patients (see section 1.6).

Atherosclerotic plaque commonly develops at the sites where major arteries branch or curve. It is there that shear force is higher and thus, shear induced platelet aggregation is initiated by the binding of soluble vWF to platelet GPIb. Advanced atheromatous lesions contains high levels of tissue factor which initiates the coagulation cascade by interacting

with factor VII and then activating factor X. Thrombin is generated as the end result of the coagulation cascade which also induces platelet aggregation. Platelet aggregates are formed from the deposited platelets which contributes to the occlusion of vessels. PDGF which is released from the platelet rich thrombus may be responsible for smooth muscle cell migration and proliferation. Mural thrombi are later incorporated into the vessel wall, which results in organised thrombi and subsequent luminal stenosis (Cassar et al. 2002).

The endothelium appears to play a pivotal role in the development of atherosclerosis through its effect on vasoregulation, platelet and monocyte adhesion and coagulation. There are an array of coronary risk factors (see Table 1.3) that may impair endothelial function **(Jagroop *et al.*, 2004)**. It is possible to predict the extent of atherosclerosis by the presence or absence of these established risk factors (Schleinitz *et al.,* 2005).

Risk factor modification has been shown to improve endothelial function. A significant reduction in cardiovascular events were observed when cholesterol and in particular LDL-C levels were reduced (Diener *et al.,* 1996).

To manage CHD it is important to distinguish the type of CHD. There are three stages of CHD, which can be characterized, as pre-clinical, stable and unstable. At the pre-clinical stage, anti-platelet therapy (ASA or warfarin), (see section 1.6) is recommended. In addition, diet modification as well as a statin is recommended for patients with an elevated cholesterol (in particular LDL-C) (2002). The second stage of CHD, the stable stage, involves the development of stable coronary symptoms. Patients are recommended diet and exercise in order to improve their lipid profile. Again, drug therapy is in the form of statins e.g. simvastatin and pravastatin (Leonardi-Bee *et al.,* 2005). Patients should receive low

dose ASA for life. In patients where ASA is contraindicated, clopidogrel can be used as a substitute (Quinn *et al.,* 1999). Patients should also receive beta-blockers. The use of angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor blockers can control blood pressure and improve survival in secondary prevention (Quinn *et al.,* 1999). The third stage of CHD, unstable CHD, is characterised by its severity and treated accordingly (Gent *et al.,* 1989). There are a further 3 stages of unstable CHD, unstable angina, acute MI and sudden ischemic cardiac death.

Table 1.3: Risk Factors for Coronary Heart Disease that can also Impair Endothelial Function

- Male gender
- Dyslipidemia
- Obesity
- Diabetes mellitus
- Hypercholesterolemia
- Tobacco/cigarette smoking
- High-fat diet
- Hypertension
- Sedentary lifestyle
- Genetics (family history of premature CHD)

Following activation, platelet responses involve PSC, aggregation and the release of several mediators (Diener *et al.,* 1996). Platelet activation has been associated with increased plasma levels of CD40 ligand (Tran *et al.,* 2004). Platelet activation *in vivo* is also associated with the physical changes such as mean platelet count, MPV and mean platelet granularity. Large denser platelets are more active in haemostasis (Leon *et al.,* 1998) (Sudlow, 2005). P-selectin is expressed following inflammation and mediates leukocyte rolling and recruitment. Moreover, increased levels of plasma P-selectin levels are associated with several cardiovascular disease (De Schryver *et al.,* 2003; **Jagroop** *et al.,* 2004; Tsiara *et al.,* 2003).

1.6 Anti-platelet Therapy

1.6.1 Clopidogrel

Clopidogrel (Plavix) is a theinopyridine, a class of ADP receptor/$P2Y_{12}$ inhibitors (Duerschmied *et al.,* 2010) and is used as a potent anti-platelet agent. It demonstrates efficacy in preventing thrombotic events (MI, stroke, and vascular death) in high risk patients (1996*, no authors listed*), (Anderson *et al.,* 2010). Clopidogrel is not active *in vivo*; biotransformation by the liver is necessary to allow the generation of an active metabolite that expresses anti-aggregatory activity (Savi *et al.,* 1992). Other ADP $P2Y_{12}$ receptor antagonists are in current clinical use or at various phases of development. These include prasugrel, ticagrelor cangrelor and elinogrel (these are further discussed in chapter 3). Trials are underway to determine if these ADP $P2Y_{12}$ receptor antagonists have a faster onset of action, and/or greater anti-thrombotic effects than clopidogrel, without an unacceptable increase in hemorrhagic or other side effects (Mousa *et al.,* 2010).

The Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (1996*, no authors listed*) study (n = 19,185) was carried out to test the efficacy and safety of a dose of 75 mg daily of clopidogrel vs ASA, 325 mg daily for 1 to 3 years. The majority of the difference in effectiveness occurred in the patients who entered the trial because of symptomatic PAD (1997b*, no authors listed*). Clopidogrel may be superior to ASA in the treatment of PAD (Matsagas and **Jagroop** *et al.,* 2002). A strong association exists between PAD and other atherosclerotic disorders such as CHD and cerebrovascular disease. Platelet hyperactivity in PAD may play a role in the process that leads to complications and disease

progression. Other studies indicated, that platelets in patients with PAD are relatively ASA-resistant (Reininger et al, 1999; 1997b *no authors listed*; Diehm *et al.,* 2004). Therefore, clopidogrel may be a better choice than ASA in treatment of PAD (Matsagas and **Jagroop** *et al.,* 2002). The data from the CAPRIE Trial, in patients with PAD, demonstrated that those randomized to clopidogrel 75 mg daily had a 24% significant (p=0.0028) reduction in vascular death, non-fatal MI, and non-fatal stroke than patients randomized to ASA 325 mg daily (1997b*, no authors listed*). These data favour the use of clopidogrel in patients with PAD. However, this interpretation is limited by the fact that this analysis was *post-hoc*. ASA alone may no longer be the optimal therapy for PAD (1997b*, no authors listed*; Diehm *et al.,* 2004).

1.6.2 Aspirin

ASA may represent the "golden balance" between yielding maximal clinical benefit with minimal complications. ASA is widely used as an effective inhibitor of platelet aggregation (1997b, *no authors listed*; Patrono, 1994). ASA works by preventing the conversion of arachidonate to TXA_2, a potent vasoconstrictor and platelet agonist, by irreversibly inactivating the cyclooxygenase (COX) activity (also referred to as COX-1 and COX-2) (Ehrman *et al.,* 1980; Roth *et al.,* 1975a). These isoenzymes catalyze the first step in prostanoid biosynthesis (i.e. the conversion of arachidonic acid to PGH_2). PGH_2 is the immediate precursors of PGD_2, PDE_2, PGF_2, PGI_2 (Ehrman *et al.,* 1980) and TXA_2 (Roth *et al.,* 1975b). In human platelets, TXA_2 is released in response to various platelet agonists (e.g. collagen, ADP, PAF, and thrombin) and induces irreversible aggregation (1997b*, no*

authors listed; Patrono, 1994). The molecular mechanism of permanent inactivation of COX activity by ASA is a consequence of the acetylation of a serine residue that prevents access of the substrate to the catalytic site of the enzyme (Loll *et al.,* 1995). Since ASA can inactivate COX-1 even in mature megakaryocytes and since 10% of the platelet pool is replaced every day, a single dose of 160 mg or the chronic administration of daily doses of 30-50 mg of ASA, is capable of completey inhibiting platelet TXA_2 production (1997b*, no authors listed*; Patrono, 1994). In contrast, the inhibition of COX-2 requires larger doses of ASA (because of decreased sensitivity of COX-2 to ASA) and a much shorter dosing interval (1997b*, no authors listed*; Patrono; 1994).

There are a few side effects associated with the use of ASA. Long-term therapy with ASA is associated with a modest increase in the incidence of upper gastrointestinal bleeding (Aster *et al.,* 2004; Weil *et al.,* 1995). The inhibition of platelet function, the impairment of PGE_2-mediated cytoprotection in the gastrointestinal (GI) mucosa, as well as the ulcerogenic effect generated by the direct contact of this drug with the gastric mucosa, contribute to the increased risk of upper GI bleeding (Liberopoulos *et al.,* 2006). Whereas the inhibition of TXA_2-mediated platelet function is dose-independent (at least for daily doses >30 mg), the side effects are dose-dependent (Patrono *et al.,* 2004a).

There is evidence that doses of approximately 300 mg/day of ASA produce fewer GI effects than those of approximately 1200 mg/day (Farrell *et al.,* 1991). The Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) investigators (Peters *et al.,* 2003) retrospectively investigated the relationship between the ASA dose (75-325 mg/daily) and the risk of major bleeding. The administration of ≤ 100 mg/day of ASA resulted in the

lowest rate of major or life-threatening bleeding complications both in placebo (1.9%) and clopidogrel (3%) arms of the trial. Bleeding risks increased with increasing ASA dose, with or without clopidogrel, without any increase in efficacy.

Although the search for the lowest effective dose of ASA for platelet inhibition was largely driven by the concern of concomitant inhibition of vascular PGI_2 production, it is still uncertain whether dose-dependent suppression of the latter attenuates the anti-thrombotic effect of ASA in the clinical setting (Patrono *et al.,* 2004a). The Anti-thrombotic Trialists' Collaboration (Diener *et al.,* 2004) meta-analysis of 287 secondary prevention studies (2002*, no authors listed*), showed that among the trials of high daily doses of ASA vs no ASA, no particular range of ASA dose was preferable for the prevention of serious vascular events. The proportional reduction in vascular events was 19% with 500-1500 mg/daily, 26% with 160-325 mg/daily and 32% with 75-150 mg/daily. However, daily doses <75 mg seemed to have a smaller effect (proportional reduction 13%). Additionally, ASA in a dose of 75 mg/day was effective enough to reduce the risk of MI or death in patients with unstable angina (1990*, no authors listed*) and chronic stable angina (Juul-Moller *et al.,* 1992), as well as to reduce the incidence of stroke or death in patients with transient ischaemic attacks (TIA) (1991*, no authors listed*). The Acetylsalicylic Acid and Carotid Endarectomy trial (Taylor *et al.,* 1999) reported that the risk of the composite outcome of MI, stroke or death within 3 months of carotid endarterectomy was significantly lower among patients taking 81 or 325 mg ASA daily than in those taking 625-1300 mg. Similarly, in the UK-TIA study, no difference in efficacy was found between doses of 300 and 1200 mg/day (Farrell *et al.,* 1991)

1.6.3 Aspirin and Clopidogrel Combination

The Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial (Yusuf *et al.,* 2001) was designed to compare the efficacy and safety of the early and long-term use of clopidogrel plus ASA with those of ASA alone, in patients (n=12,562) with acute coronary syndromes and NSTEMI. Patients who presented within 24 h after the onset of symptoms, were randomly assigned to receive either clopidogrel (300 mg immediately, followed by 75 mg once daily) or placebo, for 3-12 months. ASA, 75-325 mg/daily, was given to all patients. Death from cardiovascular causes, non-fatal MI, or stroke occurred in 9.3% of the patients in the clopidogrel group and 11.4% in the placebo group. The second primary outcome (composite of the first primary outcome or refractory ischaemia) occurred in 16.5% of the patients in the clopidogrel group and in 18.8% of the patients in the placebo group ($p<0.001$). In the Percutaneous Coronary Intervention (Mehta *et al.,* 2001) sub-study of the CURE trial (Mehta *et al.,* 2001) 2,658 patients undergoing PCI were pretreated with ASA plus clopidogrel or ASA plus placebo before PCI. The primary endpoint was a composite of cardiovascular death, MI, or urgent target-vessel revascularization within 30 days of PCI. The number of patients with the primary endpoint was significantly lower in the clopidogrel group (7.2%) compared with the placebo group (4.2%) (95% CI 0.4-0.85, $p = 0.005$). As continuity of the PCI-CURE trial, the Clopidogrel for the Reduction of Events During Observation (CREDO) trial (Steinhubl *et al.,* 2002) was designed to evaluate the efficacy and safety of clopidogrel therapy for 1 year and the efficacy and safety of a loading dose of clopidogrel prior to elective PCI.

The Clopidogrel as Adjunctive Reperfusion Therapy (CLARITY)-Thrombolysis in MI (TIMI) 28 study (Sabatine *et al.,* 2005) enrolled 3,491 patients, 18 to 75 years of age, who presented within 12 h after the onset of STEMI. Patients were randomly assigned to receive either clopidogrel (300 mg loading dose followed by 75 mg once daily) or placebo. All patients were to be treated with a fibrinolytic agent, ASA (150-325 mg on the first day and 75-162 mg/day thereafter), and for those receiving a fibrin-specific lytic agent, heparin for 48 h. Coronary angiography was performed 48-192 h after the start of study medication. The primary endpoint was the composite of an occluded infarct-related artery (defined by a TIMI flow grade of 0 or 1), death from any cause before angiography could be performed, or recurrent MI before angiography. For patients who did not undergo angiography, the primary endpoint was death or recurrent MI by day 8 or hospital discharge, whichever came first. There was a 6.7% reduction in the rate and a 36% reduction in the odds of the primary endpoint with clopidogrel therapy (95% CI, 24-47%; $p<0.001$).

Other studies that examined ASA in combination with clopidogrel, include the Clopidogrel and Metoprolol in MI Trial (1996*, no authors listed*; Chen *et al.,* 2005), the Management of Atherothrombosis with Clopidogrel in High-risk patients trial (Diener *et al.,* 2004), and the Clopidogrel and Aspirin for Reduction of Emboli in Symptomatic Carotid Stenosis (Markus *et al.,* 2005) trial (Markus *et al.,* 2005).

The CARESS trial (Diener *et al.,* 2004) was designed to evaluate whether clopidogrel in combination with ASA is superior to ASA alone in reducing the incidence of asymptomatic microembolic signals (MES) as detected by transcranial Doppler ultra-sound (TCD). This was carried out in patients with recently diagnosed symptomatic carotic

stenosis. 107 patients diagnosed with MES were randomised for treatment of either ASA or ASA plus clopidogrel. Results indicated a significant reduction in the primary end-point of 43.8 % of dual-therapy patients who were MES positive on day 7, as compared to 72.7 % of mono-therapy patients. The secondary end-point of MES frequency per hour was reduced (in comparison to baseline) by 64.1 % in the dual-therapy group at day 7, and by 61.6 % on day 2. In addition to TCD recordings, collagen-induced platelet aggregation tests were carried out on PRP (from 71 patients) at baseline and at day 7. Mean maximum intensity of platelet aggregation at day 7 was 106.7 % of baseline in the monotherapy group and 70.9 % in the dual-therapy arm (a relative risk reduction 36.6 %). Markus *et al.* concluded that in patients with recently symptomatic carotid stenosis, combination therapy with clopidogrel plus ASA is more effective than ASA alone in reducing asymptomatic embolism (Diener *et al.,* 2004).

Jagroop *et al* (Jagroop *et al.,* 2004) evaluated the effect of ASA (75 mg/day), clopidogrel (75 mg/day) and both anti-platelet drugs on platelet function in patients with PAD (see chapter 3). There was a significant decrease on ADP-induced aggregation after clopidogrel but not after taking ASA. Dual therapy significantly decreased SPA, which was not significantly altered by either monotherapy. The same phenomenon was observed with 5HT-induced aggregation. Soluble P-selectin decreased significantly with the combination therapy.

A cost-effectiveness analysis of combination therapy (ASA plus clopidogrel) showed that in patients with high risk acute coronary syndromes, 1 year of treatment with

clopidogrel plus ASA results in greater life expectancy than ASA alone, at a cost within the traditional limits of cost-effectiveness (Schleinitz *et al.,* 2005).

1.6.4 Aspirin and Dipyridamole Combination

The combination of ASA with dipyridamole (DP) was compared with ASA monotherapy for stroke prevention among patients with TIA or stroke, in several clinical trials. The European Stroke Prevention Study-2 (ESPS-2 study) (Diener *et al.,* 1996) evaluated this anti-platelet combination therapy in 6,602 patients with TIA or stroke in the preceding 3 months. After 2 years of follow-up, the combination therapy produced a relative risk reduction by 23.1% compared with ASA alone, in both the stroke endpoint and the composite stroke or death end-point. The ESPS-2 trial was analyzed together with additional trials included in the meta-analysis by the ATC, the addition of DP to ASA was associated with a non-significant 6% risk reduction in serious vascular events (fatal stroke, MI, vascular deaths) compared with ASA monotherapy (Tran *et al.,* 2004).

The MATCH trial set out to determine whether the addition of aspirin to clopidogrel could have a greater benefit than clopidogrel alone in prevention of vascular events with potentially higher bleeding risk (Diener *et al.,* 2004). A randomised, double-blind, placebo-controlled trial to compare aspirin (75 mg/day) with placebo. This was carried out in 7599 high-risk patients (treated for 18 months) with recent ischaemic stroke or transient ischaemic attack, and at least 1 additional vascular risk factor who were already receiving clopidogrel 75 mg/day (Diener *et al.,* 2004). The results from this study indicated that patients at high-risk (i.e. who recently suffered ischaemic stroke or transient ischaemic attack, TIA), when

treated with a combination of aspirin and clopidogrel are associated with a non-significant difference in the reduction of major vascular events. Moreover, addition of aspirin increases the risk of life-threatening or major bleeding (Diener *et al.,* 2004).

1.6.5 Aspirin and Ticlopidine Combination

In the STent Anti-coagulation Restenosis Study (STARS) (Leon *et al.,* 1998) 1,965 patients undergoing coronary artery stenting were randomized to 1 of 3 different anti-thrombotic regimens: 325 mg/day of ASA, ASA plus ticlopidine 250 mg twice daily, or ASA plus warfarin daily to achieve anincidence of neutropenia or thrombocytopenia of 2-2.5. The main endpoints of this trial consisted of clinical and angiographic cardiac outcomes within 30 days of coronary stenting, and included MI and a composite outcome of MI, vascular mortality, vessel thrombosis and need for revascularization. The combination of ASA with ticlopidine was more effective than ASA alone or ASA plus warfarin for the MI and composite outcomes, but resulted in more major haemorrhages, defined as any haemorrhage requiring transfusion. Ticlopidine has been linked with aplastic anemia (Bortolotti *et al.,* 2002; Kao *et al.,* 1997; Symeonidis *et al.,* 2002) and is discussed further in section 1.6.7

1.6.6 Dipyridamole

Dipyridamole (DP) is a pyrimidopyridine derivative with anti-platelet and vasodilator properties. Several possible anti-platelet actions of DP include the inhibition of platelet cyclic nucleotide phosphodiesterase, the direct stimulation of PGI_2 release from endothelial cells and the inhibition of adenosine uptake by platelets (Sudlow, 2005). All of

these putative mechanisms increase intra-platelet adenosine 3',5'-cyclic monophosphate (cAMP), which inhibits the mobilization of free calcium, central to platelet activation. DP is also a potent vasodilator, and its coronary dilating effect is the reason for its use in diagnostic stress echocardiography and thallium imaging. During rapid intra-venous administration in these procedures, DP tends to lower blood pressure. However, the long-term (>15 months) oral administration of DP in patients with recent cerebral ischaemia of arterial origin, did not affect blood pressure (De Schryver, 2003).

In the ESPS-2 study (Diener *et al.,* 1996), DP compared with placebo, significantly reduced the incidence of stroke (p=0.04). In a *post-hoc* analysis of ESPS-2 data, treatment with DP in patients with coronary heart disease or MI at study entry, did not result in a higher number of fatal and non-fatal cardiac events.

The ATC's meta-analysis (2002*, no authors listed*) of direct randomized comparisons between DP alone and ASA alone in high risk patients, found no significant difference in effect on serious vascular events, including stroke, MI, or vascular death. However, since the largest body of evidence concerns ASA, and the wide confidence interval includes the possibility that DP is less effective than ASA, this implies that DP alone should not generally be considered as an alternative to ASA.

In a meta-analysis by the Dipyridamole in Stroke Collaboration (DISC) (Leonardi-Bee *et al.,* 2005) in patients with previous ischaemic stroke or TIA, DP significantly reduced stroke compared with controls (OR 0.82; 95% CI, 0.68-1.00). Furthermore, this meta-analysis showed that the combination of DP with ASA significantly reduced stroke compared with DP alone (OR 0.74; 95%CI, 0.60-0.90). Dual therapy also significantly

reduced the composite outcome of non-fatal stroke, non-fatal MI, and vascular death as compared with DP alone (OR 0.76; 95% CI, 0.64-0.90).

1.6.7 Ticlopidine

Ticlopidine has been used as an inhibitor of platelet aggregation and was widely used in PAD. However, the use of this drug is limited since it has been associated with neutropenia and other adverse hematologic effects, such as aplastic anaemia (Bortolotti *et al.,* 2002; Kao *et al.,* 1997; Symeonidis *et al.,* 2002). Aplastic anaemia is a rare complication that carries high mortality (Bortolotti *et al.,* 2002). A meta-analysis of the literature evaluated 57 patients who were on ticlopidine (Symeonidis *et al.,* 2002). A reversible direct cytotoxic effect of ticlopidine on the pluripotent/bipotent hematopoietic progenitor stem cell was proposed with the use of ticlopidine (Symeonidis *et al.,* 2002). It was estimated that ticlopidine-induced aplastic anaemia was higher than previously suspected (Symeonidis *et al.,* 2002).

To summarize, there is currently evidence regarding oral anti-platelet treatment in patients with CVD, CAD, and PAD. It can be stated, that aspirin, ticlopidine, or clopidogrel on its own, and aspirin combined with clopidogrel, or aspirin combined with dipyridamole are effective in preventing recurrent vascular events among various subgroups of patients with vascular disease (Tran *et al.,* 2004). Moreover, current clinical trial evidence prefers the use of aspirin or clopidogrel as first-line agents for the majority of patients with vascular disease. The future practice by clinicians will be dictated by further clinical trials evaluating combination anti-platelet therapies.

1.6.8 GPIIb/IIIa Receptor Antagonists

One of the mechanisms to inhibit platelets is to block the platelet surface membrane glycoprotein (GP) IIb/IIIa receptor, which binds circulating fibrinogen or vWF and crosslinks platelets as the final common pathway to platelet aggregation (Auer *et al.,* 2003). Intravenous agents directed against this receptor include the chimeric monoclonal anti-body fragment abciximab, the peptide inhibitor eptifibatide, and non-peptide mimetics tirofiban and lamifiban (Auer *et al.,* 2003). Clinical trials with a combined total of more than 18,000 patients have shown the benefits of intra-venous IIb/IIIa blockade (i.e. the final common pathway for aggregation) (Mak *et al.,* 1999b). Overall, at 30 days, 13 fewer deaths or MIs occurred for every 1000 patients treated in these trials. This favorable outcome was extended to 6 months, resulting in 16 fewer such events per 1000 patients treated. Over 20,000 patients were enrolled in 9 (1994*, no authors listed*) major studies of abciximab, eptifibatide, and tirofiban (1994 *no authors listed*; 1997b*, no authors listed*). The result was that many countries approved the use of abciximab due to the EPIC trial (1994*, no authors listed)*, eptifibatide and tirofiban showed positive trends in the IMPACT-II trial (1997b*, no authors listed*). Despite being as potent as their intra-venous counterparts, all of the oral inhibitors showed no benefit or even increased mortality in clinical trials (Cox, 2004b). There are a number of possible reasons for their failure. The target was different, chronic treatment to prevent thrombotic events as opposed to short-term treatment to prevent acute

events and as a result, different dosing regimens were used (Cox, 2004b). Many of the oral inhibitors had low bioavailability that led to a large peak-trough difference (Storey, 2002). Tirofiban is discussed further in Chapter 5.

1.6.9 Picotamide

Picotamide, a derivative of methoxy-isophtalic acid, is an antagonist of TXA_2/PGH_2 receptors, which also inhibits TXA_2 synthase at equivalent concentrations. This dual action may enhance the therapeutic efficacy in the prevention of thrombosis, including inhibition of platelet aggregation and accumulation of anti-aggregatory prostaglandins (Gersele et al. 1991). In a double blind, placebo-controlled study in 2,313 patients with PAD, picotamide significantly reduced the relative risk of combined major and minor cardiovascular events by 23% compared to placebo (Balsano et al. 1993). The Drug evaluation in Atherosclerotic Vascular disease In Diabetics (DAVID) study included 1,209 adults, aged 40-75 years with type 2 diabetes and PAD (Neri Sereneri et al. 2004). Patients were randomized to receive picotamide (600 mg twice daily) or ASA (320 mg/day) for 24 months. The cumulative incidence of the 2 years overall mortality was significantly lower in the picotamide group (3.0%) compared with the ASA group (5.5%) (RRR 0.55 for picotamide vs ASA).

1.6.10 Cilostazol

Cilostazol is a selective inhibitor of phosphodiesterase-III (PDE-III) with anti-platelet, anti-thrombotic and vasodilating properties (Chapman *et al.,* 2003; Robless *et al.,* 2008). Cilostazol exhibits anti-proliferative effects on smooth muscle cells and has

beneficial effects on high-density lipoprotein-cholesterol and triglyceride levels. The efficacy of cilostazol was demonstrated in randomized, double blind trials, where the administration of the drug in over 2,000 patients with moderate to severe intermittent claudication (IC), resulted in a significant increase of walking distance and in an improvement of quality of life (Chapman *et al.,* 2003).

The Cochrane Peripheral Vascular Diseases Group carried out searches to determine the effect of cilostazol on improving walking distance and in reducing vascular mortality and cardiovascular events in patients with stable IC (Robless, 2008; Storey, 2002). The authors concluded that patients with IC should be treated with secondary prevention for cardiovascular disease and reported that cilostazol had a beneficial effect in improving walking distance in people with IC (Robless, 2008; Storey, 2002). Moreover, cilostazol is generally well tolerated, but common adverse events are headache, diarrhoea, abnormal stools, rhinitis and peripheral oedema. Co-administration of cilostazol with ASA or warfarin does not influence coagulation parameters, bleeding time or platelet aggregation (Barnett *et al.,* 2004).

1.6. 11 Sarpogrelate

Sarpogrelate is a selective $5HT_{2A}$ receptor antagonist that has antiplatelet, antithrombotic, anti-atherosclerotic and anti-anginal activity (Saini *et al.,* 2004). In human beings, dogs, monkeys and rats, when given orally, sarpogrelate hydrochloride is first metabolized to (R,S)-1-[2-[2-(3–methoxyphenyl)ethyl]phenoxy]-3-(dimethylamino)-2-

propanol-M-1 (Maurer-Spurej *et al.,* 2001) by hydrolysis of its succinic ester moiety (Saini *et al.,* 2004).

There are a number of reports that support the importance of 5HT in the pathogenesis of atherothrombosis (Ban *et al.,* 2007; Doggrell, 2004; Nishihira *et al.,* 2006). 5HT is stored in the dense granules of platelets and is secreted during platelet activation to stimulate smooth muscle proliferation, vascular contraction, potentiates thrombus formation and causes vessel occlusion (Nishihira *et al.,* 2006).

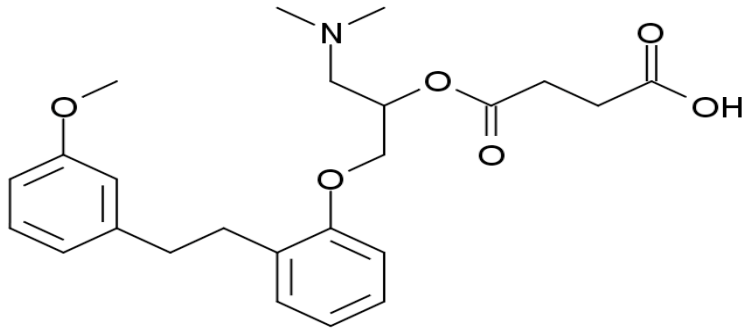


Figure1.8: Molecular structure of sarpogrelate

Furthermore, Barradas *et al*. (Barradas *et al.,* 1988) as well as others (Ban *et al.,* 2007; Wiernsperger, 1990) demonstrated that plasma 5HT levels are higher in patients with diabetes mellitus and PAD, thereby providing another line of clinical evidence that 5HT is involved in the development of atherothrombosis (Uchiyama *et al.,* 2007).

5HT alone is a mild platelet agonist that only induces shape change and reversible aggregation (**Jagroop** *et al.,* 2000d; **Jagroop** *et al.,* 2000a; **Jagroop** *et al.,* 2001; Qi *et al.,* 1996). Moreover, 5HT synergistically amplifies platelet aggregation induced by ADP,

collagen or epinephrine (Hara *et al.,* 1991). Therefore, the 5HT receptor could be a good target for antiplatelet and antithrombotic therapy. Thus, the effect of 5HT receptor antagonists have been evaluated by using combinations of the platelet agonists (**Jagroop** *et al.,* 1998; **Jagroop** *et al.,* 2000b; **Jagroop** *et al.,* 2001;Qi *et al.,* 1996).

Furthermore, sarpogrelate a selective $5HT_{2A}$ receptor antagonist has been developed as an inhibitor of platelet aggregation, and vasoconstriction induced by 5HT and has been the subject of investigation. Thus, sarpogrelate was shown to inhibit two combinations of agonists (0.5 μmol/l 5HT plus 3 μmol/l epinephrine, and 1 μmol/l 5HT plus 3 μmol/l epinephrine)– induced platelet aggregation in a dose dependent manner ($P < 0.025$) in patients with ischemic strokes (Uchiyama *et al.,* 2007). The effect of sarpogrelate on endothelial function in patients with PAD was investigated by Miyazaki *et al* (Miyazaki *et al.,* 2007) who suggested that long-term oral administration of sarpogrelate improves vascular function in these patients. The effect of sarpogrelate on platelet function in patients with Buerger's disease was investigated (Rydzewski *et al.,* 1996). The rational was that these patients are more sensitive to 5HT, and spontaneously release more 5HT with decreased uptake of this amine (Nishikimi *et al.,* 1992). Thus, treatment with sarpogrelate (100 mg, p.o., 3 times a day) for 4 weeks showed a significant decrease in plasma 5HT in damaged microvasculature, an effect which also lasted 8 weeks. In addition, sarpogrelate showed to improve the ankle pressure index (0.85 ± 0.18 vs 0.79 ± 0.21 before treatment) after treatment for 8 weeks, in patients with Buerger's disease (Rydzewski *et al.,* 1996).

The Sarpogrelate-Aspirin Comparative Clinical Study for Efficacy and Safety in Secondary Prevention of Cerebral Infarction (S-ACCESS), was a randomized double-blind

study of sarpogrelate (selective 5-HT_{2A}) receptor antagonist) versus aspirin in 1,510 Japanese patients (Shinohara *et al.,* 2008). It was reported that S-ACCESS failed to demonstrate non-inferiority of sarpogrelate to aspirin for preventing the recurrence of cerebral infarction. No specific baseline characteristic resulting in a significant difference between the effects of sarpogrelate and aspirin was identified. Aspirin was superior in most subgroups, except diabetics. However, sarpogrelate may be a useful treatment option for Japanese patients with diabetes (Shinohara *et al.,* 2009).

1.6.12 Nitroaspirin

The coupling of Non Steroidal Anti-inflammatory Drugs (NSAIDs) to appropriate chemical spacers bearing a nitric oxide (NO)-donating moiety, led to the birth of a new class of chemical entities, called NO-NSAIDs (Nitric Oxide Non Steroidal Anti-Inflammatory Drugs) or CINODs (Cyclooxygenase-Inhibiting Nitric Oxide Donors) (Gresele *et al.,* 2006; Momi *et al.,* 2000). Nitroaspirin (NCX 4016) is one of the leading compounds in this new class of drugs. NCX 4016 (2-acetoxybenzoate 2-[1-nitroxy-methyl]-phenyl ester) was synthesized by the ester linkage of a NO-releasing moiety to aspirin at NicOx SA Research Institute (Milan, Italy). The acetylsalicylic acid molecule is linked at the meta position to a chemical spacer (hydroxybenzylalcohol) bearing the NO-donating moiety (benzenemethanol-3-hydroxy-a-nitrate or NCX 4015) and the resulting molecule has a molecular mass 1.8 fold greater than that of aspirin (MW 331.28). The NO-NSAIDS were developed in an attempt to obtain safer and potentially more active compounds than the parent drugs (Gresele *et al.,* 2003; Gresele *et al.,* 2006; Momi *et al.,* 2000).

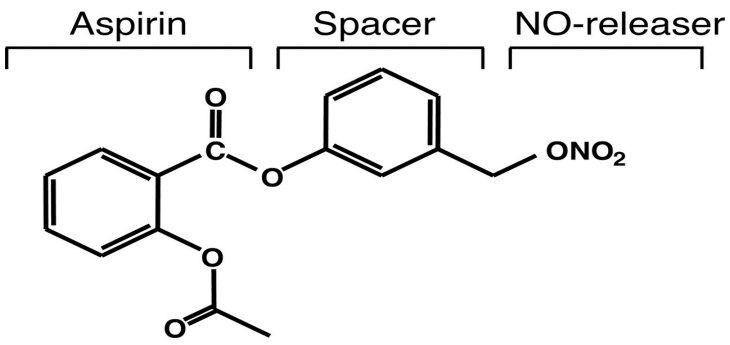


Figure 1.9: Molecular structure of nitroaspirin

Aspirin inhibits platelet aggregation by acting as a COX inhibitor and thus suppressing the production of TXA_2, an important agonist of platelet activation and a vasoconstrictor (Patrono, 1994). However, the suppression of only one pathway of platelet activation, albeit important, may represent a limitation for effectiveness of aspirin (Gresele *et al.,* 1991). These considerations have lead to the search for new drugs that could suppress platelet activation more effectively than aspirin.

NO plays and significant role in preventing vasoconstriction stimulated by platelet released mediators at localised sites of vessel wall injury in coronary arteries (Lechi *et al.,* 1996a) and is also a powerful platelet inhibitor that can suppress platelet adhesion and activation induced by a wide range of agonists (Loscalzo, 2001). NCX 4016 the agent that combines a NO-donating moiety with aspirin was shown to exert a number of anti-inflammatory activities beyond that of aspirin, to reduce ischemia/reperfusion damage, to correct endothelial dysfunction and to prevent oxygen radicals induced vascular remodelling in animal models (Gresele *et al.,* 2003; Momi *et al.,* 2005). The anti-thrombotic activity of NCX 4016, was examined *in vivo* in two different animal models (mice and rabbits) of

platelet dependent and independent pulmonary thromboembolism and compared with that of aspirin. The findings from this study demonstrated that NCX 4016 exerted a more pronounced anti-thrombotic activity than aspirin largely due to deeper inhibitory effects on platelets (Momi *et al.,* 2000).

The antiplatelet activity of NCX 4016 as well as that of its chemically related NCX 4215 were studied *in vivo.* Both drugs inhibited AA-induced platelet aggregation and TXA_2 production in a dose dependent manner and NCX 4016 proved to be significantly more potent than NCX 4215 (Lechi *et al.,* 1996b).

The effects of aspirin with those of NCX 4016 in preventing the acute, systemic endothelial dysfunction provoked by exercise-induced ischemia of lower limbs in patients with intermittent claudication was studied. After 4 weeks treatment, the impairment of flow-mediated vasodilation induced by exercise was still present in the aspirin treated group while it was abolished in the NCX 4016-treated group. This study concluded that a NO-donating aspirin, but not aspirin alone, prevents effort-induced endothelial dysfunction (Gresele *et al.,* 2007).

1.6.13 Trapidil

Trapidil, an anti-platelet drug, has been shown to reduce restenosis after angioplasty. It exerts its action, at least in part, by inhibiting vascular smooth muscle cell proliferation and antagonizing PDGF. PDGF is activated in response to vascular injury and plays a significant role in SMC proliferation, chemotaxis of inflammatory cells, and the formation

of extracellular matrix. Trapidil is currently used as a coronary artery vasodilating agent and is also used for the prevention of ischemic symptoms of cerebral vasospasm.

The Studio Trapidil versus Aspirin nella Restenosis Coronarica (STARC) was a multi-centre, randomized, double blind trial to assess the effects of trapidil in angiographic restenosis prevention after percutaneous transluminal coronary angioplasty (PTCA). Trapidil reduced restenosis after PTCA at the dosage of 100 mg TID and favourably influences the clinical outcome thereafter (Maresta 1994).

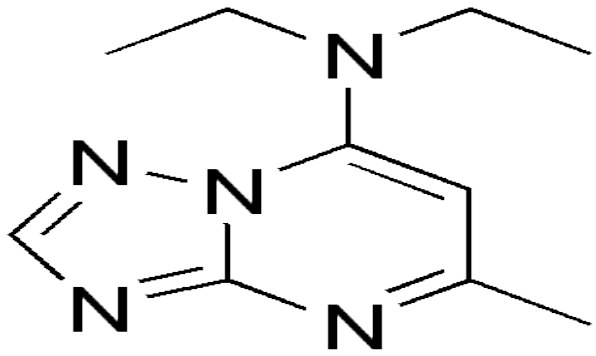


Figure 1.10: The molecular structure of trapidil

Another study (JOVIN, 2006) investigated whether trapidil can inhibit the aggregation of platelets from heart transplant recipients. These patients show platelet hyperaggregability, which may be related to the incidence of graft vasculopathy, and is not corrected by aspirin. Platelet count, MPV, and adenosine diphosphate (ADP)-induced platelet aggregation were determined in 18 heart transplant recipients and 12 healthy subjects. The trapidil-treated samples showed significantly decreased platelet aggregation compared with the control samples (24.2 ± 12.6 vs $66.7 \pm 11.7\%$; $P < 0.001$). Platelets from

heart transplant recipients showed an increased MPV and increased ADP-induced aggregation. Trapidil effectively reduced the ADP-induced aggregation ex vivo.

To investigate the effect of long-term trapidil on the prognosis of patients with CHD, a study was carried out with a mean follow up of 924 days (Hirayama *et al.,* 2003). A total of 1,743 patients who showed angiographic evidence of >25% stenosis in any coronary artery was investigated. Patients were treated with trapidil (n=873, 100 mg, 3 times/day) and compared with a group who did not receive trapidil (n=870). The study concluded the long-term intervention with trapidil in CHD reduces the incidence of cardiovascular events and improves the prognosis of CHD patients (Hirayama *et al.,* 2003).

1.6.14 Pentoxifylline

Pentoxifylline (Trental), 3,7-dimetyl-1(5′-oxo-hexyl)xanthine is a haemorheologic drug used in the treatment of PAD. This drug works by decreasing blood viscosity, stimulating prostacyclin formation, increasing blood flow by increasing flexibility of RBCs, decreasing RBC hyperaggregation, reducing platelet aggregation and decreasing fibrinogen concentration (Moher *et al.,* 2000; Samlaska *et al.,* 1994).

In as early as 1977, pentoxifylline was shown to be a powerful inhibitor of ADP and 5HT-induced platelet aggregation of monkey platelets *in vivo* (Gastpar *et al.,* 1977). Platelet aggregation studies in human PRP confirmed that pentoxifylline inhibited 5HT- as well as epinephrine- induced platelet aggregation in a dose dependent manner (Nenci *et al.,* 1981). Pentoxifylline is metabolised in humans to at least 7 metabolites (Magnusson *et al.,* 2008). Magnusson *et al*. investigated the relative potencies of pentoxifylline and its metabolites,

M1, M4 and M5 to inhibit platelet aggregation in human whole blood. They concluded that: in the following potency order R-M1, rac-M1, pentoxifylline, S-M1 and M4 has significant effects on platelet aggregation in whole blood *in vivo*. The main *in vivo* effect, however, was caused by S-M1 and pentoxifylline (Magnusson *et al.,* 2008).

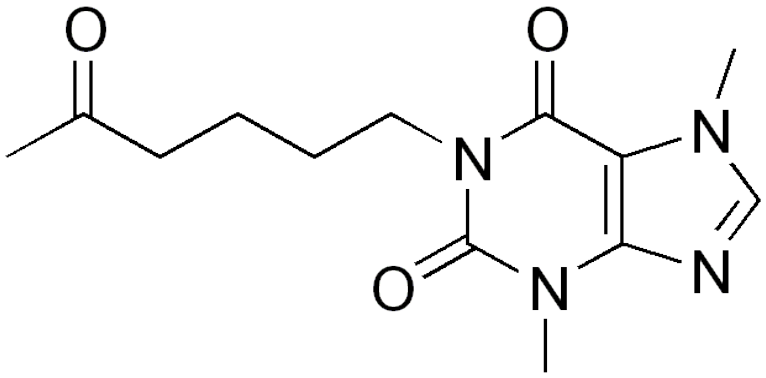


Figure 1.11: The molecular structure of pentoxifylline

In hypertensive type 2 DM patients, pentoxifylline exerted a significant anti-aggregatory effect in a dose dependent decrease in clot retraction *in vivo* but there was no significant change in *ex vivo* clot retraction. However, there were improvements in inflammatory markers, oxidative stress and platelet-aggregation by pentoxifylline (Maiti *et al.,* 2007). In contrast, another study that compared pentoxifylline with cilostazol, failed to corroborate the reports that pentoxifylline improves erythrocyte membrane deformability and decreases viscosity in patients with intermittent claudication. However, this may be explained by the limitation of the small number (n=20 in each group) of patients who took part in the study (Dawson *et al.,* 2002). Indeed, in a larger randomised, double blind, placebo-controlled trial, cilostazol therapy was associated with significantly greater improvement in walking distance compared with pentoxifylline and placebo (which both

exhibited similar effects) (Dawson *et al.,* 2000). Thus, having investigated treatment of pentoxifylline on patients with intermittent claudication, attention was directed towards more difficult patient population, i.e. those with critical limb ischemia. A multi centre, double blind, placebo-controlled study of intra-venous pentoxifylline in the acute management of critical ischemia was carried out, by the European Study Group. Results showed that a significant reduction in rest pain with pentoxifylline. They suggest the urgent need for further study of medical management of patients with critical ischemia, possibly comparing medical and surgical treatment (1995, *no authors listed*).

1.6.15 Beraprost

Beraprost sodium (beraprost) is a stable, orally active prostacyclin (Ehrman *et al.,* 1980) analogue with pharmacodynamic properties similar to those of PGI_2 (Melian *et al.,* 2002). The mechanisms of its interactions are likely to involve relaxation of vascular smooth cells, inhibition of platelet aggregation, and dispersion of platelet aggregates, inhibition of chemotaxis and cell proliferation, inhibition of production and secretion of endothelin and cytoprotective effects (Nishio *et al.,* 2001; Vane *et al.,* 1995).

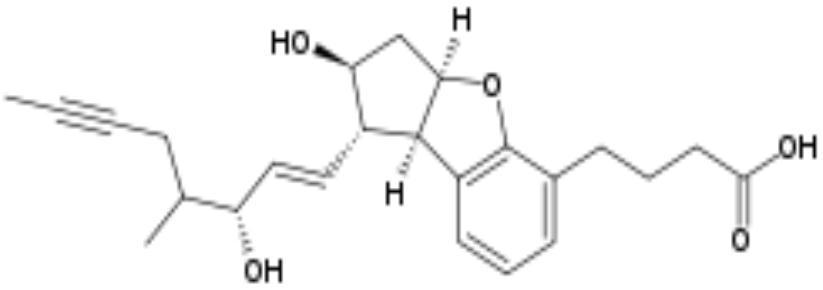


Figure 1.12: The molecular structure of beraprost

Beraprost binds to PGI_2 receptors on the platelet, thereby activating adenylate cyclase and increasing cyclic adenosine and guanosine monophosphate levels (cAMP and cGMP, respectively) in the platelet membranes of humans, dogs and rats (Kajikawa *et al.,* 1989). It has been suggested, that the effect of beraprost on cAMP and cGMP inhibits the release of Ca^{2+} from intra-cellular storage sites reducing the transmembrane influx of $Ca^{2+.}$ Moreover, this reduction appears to cause relaxation of the smooth muscle cells thereby inducing vasodilation (Melian *et al.,* 2002).

PGI_2, an endogenous prostaglandin, is the main product of AA, primarily produced in endothelial cells (and to lesser extent in smooth muscle cells) of all vascular tissues (Weksler *et al.,* 1977). PGI_2 is the most potent endogenous inhibitor of platelet aggregation and causes dispersion of existing platelet aggregates *in vivo* as well as in the systemic circulation. PGI_2 is involved in complex interactions of the vessel wall, blood flow and platelet function. It has been hypothesised, that PGI_2 antagonises the platelet aggregation and vasoconstriction caused by endogenous TXA_2 (Sugawara *et al.,* 2010; Vane *et al.,* 2003).

Beraprost has been indicated for use in Buerger's disease and arteriosclerosis obliterans, PAD, pulmonary arterial hypertension, Reynaud's phenomenon. In an animal model (rat) beraprost was shown to improve blood flow and walking disturbances associated with arterial occlusion (Miyamoto *et al.,* 2006). In patients with arteriosclerosis obliterans who experienced intermittent claudication, beraprost tended to improve walking distance and relieve symptoms. In addition, there was a decrease in endothelin-1 levels which was measured in this study to assess the mechanism of action of beraprost (Matsumoto *et al.,*

2010). In contrast, another study (Mohler, III *et al.,* 2003) found that beraprost was not an effective treatment to improve symptoms of intermittent claudication in patients with PAD. They did however, recommend confirmation in a larger prospective investigation (Mohler, III *et al.,* 2003).

1.6.16 Parogrelil

Parogrelil (NM-702), 4-bromo-6-[3-(4 chlorophenyl) propoxy]-5-[(pyridin-3-ylmethyl) amino]pyridazin-3(2H)-one hydro chloride was synthesized as a potent vasodilatory and antiplatelet agent (Ishiwata *et al.,* 2007). There are currently 2 phosphodiesterase (PDE) inhibitors, cilostazol (see section 1.6.10) and pentoxifylline (see section 1.6.14) that are approved for the treatment of IC in several countries including the US and the UK (Ishiwata *et al.,* 2007). This novel phosphodiesterase (PDE) inhibitor NM-702, was also being investigated for its use in the treatment of patients with PAD (Brass *et al.,* 2006). NM-702 showed improvement in laboratory- and ambulatory-based exercise performance in patients with claudication after being treated for 24 weeks (Brass *et al.,* 2006).

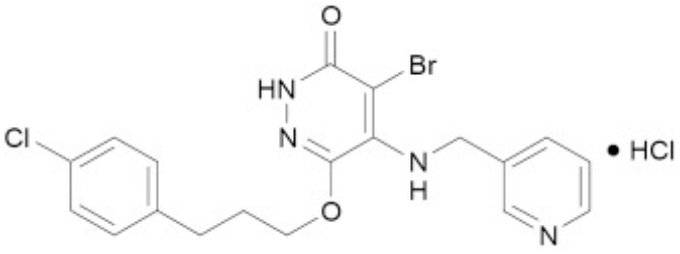


Figure 1.13: The molecular structure of parogrelil

PDEs are important in cell signaling by hydrolysing cAMP and cGMP. The potency and selectivity of NT-702 against human PDEs is uncertain. However, in a study using rhPDE1 to PDE6, it was observed that NT-702 selectively and potently inhibited PDE3. The selectivity of NT-702 to PDEs was similar to that of cilostazol (Sudo *et al.,* 2000). These findings were supported by the results from a basic pharmacological study where NT-702 selectively inhibited PDE3 (and PDE5) purified from rabbit heart and platelets (Ikegawa *et al.,* 1995). Ishiwata *et al* showed that NT-702 inhibited human platelet aggregation induced by multiple agonists. NT-702 also inhibited phenylephrine-induced vasoconstriction of the rat aorta. The authors propose that this may be due to the potent PD3A inhibitory activity of the compound (Ishiwata *et al.,* 2007). Moreover, in comparison to cilostazol, NT-207 inhibited platelet aggregation 250 – 520 times more potently and demonstrated vasorelaxation activity 42 times more. They suggested that NT-702 improved peripheral circulation of ischaemic hind limb and the reduced walking performance through the potent antiplatelet and vasorelaxant activities that can be explained by at least PDE3A inhibition (Ishiwata *et al.,* 2007).

In addition to its anti-platelet and vasorelaxant actions, NT-702 also appeared to have anti-inflammatory as well as bronchodilating effects when investigated on the asthmatic responses in guinea pigs (Hori *et al.,* 2009). Thus, it has been proposed that NT-702 might be useful as a novel potent therapeutic agent, for the treatment of bronchial asthma, and benefits from having both bronchodilating and anti-inflammatory effects (Hori *et al.,* 2009).

It is not clear as to which mechanisms are involved in dilation responses with NT-702. However, findings from a study showed that NT-702 dose-dependently dilated isolated spinal arterioles of rabbits via endothelium-dependent and endothelium-independent mechanisms (Mizuno *et al.,* 2008). In addition, the authors put forward the theory that protein kinase A, and tetraethylammonium sensitive k^+ channels, may be involved in the NT-702-induced vasodilation (Mizuno *et al.,* 2008). Further studies are needed to fully understand the mechanisms of NT-702 and its use in other diseases.

1.6.17 Unfractionated Heparin and Low Molecular Weight Heparins

It is of interest that agents with anti-thrombin activity may inhibit thrombin-induced platelet activation. For example, we previously showed that the low molecular weight heparin (LMWH), nadroparin (**Jagroop** *et al.,* 1996) added *in vivo* (**Jagroop** *et al.,* 1996) inhibits PSC and platelet aggregation**.** The inhibitory action of nadroparin was specific to thrombin since this LMWH did not influence PSC induced by other agonists. In whole blood, unfractionated heparin (UH) but not nadroparin, significantly enhanced spontaneous platelet aggregation and increased MPV when compared with LMWH. We concluded that LMWH, nadroparin, exerts less platelet activation than UH (**Jagroop** *et al.,* 1996; **Jagroop** *et al.,* 2007). It is noteworthy to mention here, that UH and LMWH appear to produce their anticoagulant effect through simultaneous inactivation of thrombin and factor X (Saucedo, 2010). In contrast, a more targeted agent that inhibits thrombin directly is bivalirudin. There was a reduced risk of bleeding associated with bivalirudin when trials compared it with

GPIIb/IIIa inhibitors (Lincoff, 2003) Tirofiban a GPIIb/IIIa inhibitor is discussed further in chapter 5.

In summation, the anti-platelets agents discussed in this chapter, significantly reduces the risk of vascular events. However, there is still some debate as to the possibility of agent superiority and the value of combination therapy. Moreover, there is a need to define optimal anti-platelet therapy for various conditions and in primary prevention. For example, what is the ideal treatment in patients at high risk of stroke, those with PAD and after vascular surgery or endovascular procedures? More research is needed to definitively answer these questions. Even then, the issue of the cost of treatment will remain paramount. Anti-coagulants are not discussed in more detail since they are not directly relevant to this thesis.

1.7 Purinergic Receptors

Purinergic receptors are present on cell surfaces and are important since signaling molecules, termed purines (adenosine, ADP and ADP) and pyrimidines (UDP and UTP) mediate diverse biological effects via these receptors (Burnstock, 2007). Currently, the sub-classifications of purines and pyrimidine receptors are based on cloning, transduction mechanisms and pharmacology (Burnstock and Kennedy 1985; Burnstock, 1996). Thus, they are characterized as follows: the P1 (adenosine) receptor family has 4 subtypes, while the P2 (ATP, ADP and UTP) receptor family has been divided into P2X ionotropic receptors (7 subtypes, $P2X_1$ – $P2X_7$), and P2Y metabotrophic G protein-coupled receptors (8 subtypes) (Burnstock 2006).

Four different types of P1 receptor subtypes have been cloned and characterized (A_1, A_{2A}, A_{2B} and A_3). They are all members of the rhodopsin-like family of the G protein-coupled receptors (Yaar *et al.,* 2005; Olah *et al.,* 2000). Transgenic knockout mice were used to demonstrate the diverse physiological effects that are mediated by the different P1 receptor subtypes. These properties include their role in the modulation of the cardiovascular system as well as the immune and central nervous systems. (Ledent *et al.,* 1997, Sun *et al.,* 2001). Null mice have been generated for each of the A_1, A_{2A}, and A_3 receptors. It was found, that in all knockout animals generated, the P1 receptors in question did not appear to play an important role during development (Lankford *et al,.* 2006). In addition, knockout mice have not yet been described for the A_{2B} receptor subtype. It is noted, that P1 and P2Y receptors are frequently expressed in the same cells (Burnstock 2007)

The cloning and functional expression of a brain G-protein-coupled ATP receptor was identified as the $P2Y_1$ purinoceptor (Webb *et al.,* 1993). A $P2Y_2$ receptor was cloned and characterized at about the same time, and its activation by either ATP or UTP was shown to elicit the mobilization of intracellular calcium (Lustig *et al.,* 1993). Thus, presently the 8 subtypes of P2Y purinoceptors are as follows: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (Burnstock 2007).

There are 2 landmark papers which cloned and characterized $P2X_1$ and $P2X_2$ receptors (Brake *et al.,* 1994; Valera *et al.,* 1994). In 1994, Valera *et al.* cloned a complementary DNA encoding the P2X receptor from rat vas deferens and expressed it in Xenopus oocytes and mammalian cells. P2X receptors have been characterized on smooth cells, autonomic and sensory neurons, where they mediate membrane depolarization and in

some cases Ca^{2+} entry (Valera *et al.,* 1994). The structure of the P2X receptor, suggest that the protein (399 amino acids long) is mainly extracellular and contains 2 transmembrane domains plus a pore-forming motif which resembles that of potassium channels. Thus, the P2X receptor defines a new family of ligand-gated ion channels (Brake *et al.,* 1994; Valera *et al.,* 1994). The new ATP-gated channels belong to an entirely different class of transmembrane signaling proteins. Members of this family include Na^+, K^+ and mechanosensory channels, which monitor and regulate changes in cell volume, shape and membrane potential. It was suggested, that a cloned P2X receptor may also play a role in cell death (Brake *et al.,* 1994). It remains to be seen if further purinergic receptors will be discovered and classified.

In this thesis the focus was on the roles of the $P2Y_1$, $P2Y_{12}$ and $P2X_1$ receptors on platelet activation (see chapter 4).

CHAPTER 2

General Methods

2.1 Selection of Subjects and Collection of Samples

Healthy volunteers who donated blood for the studies in this thesis were either members of staff or students of the Royal Free & University College Medical School, Royal Free campus, London, UK. All denied taking any drugs for at least 2 weeks prior to sampling. All volunteers were adults aged above 18 years at the time of sampling. Where appropriate the ages and genders of the volunteers are described, and written informed consent was attained.

The department has permission from the Royal Free Hospital Ethical Committee to take small (20 ml) blood samples from healthy volunteers. These sampling were repeated at intervals of less than two months from the same individual

Venous blood was collected in 3.8% tri-sodium citrate (1 part citrate to 9 parts blood), by venepuncture from an antecubital vein. Large vessels were used to ensure good blood flow and minimum stasis. Blood was obtained with a 21G butterfly in 10 or 20 ml plastic disposable and sterile syringes. All universal precautions were used during sample collection. In order to minimise platelet activation the whole blood was gently poured down the side of the tube containing citrate, and then carefully inverted 4-5 times to allow total mixing of the anti-coagulant (*see Appendix A & B: reference lists authored by **IA Jagroop**)*.

2.2 Preparation of Platelet Rich Plasma

The citrated blood was centrifuged at x160 g for 15 min at room temperature. The PRP was then carefully collected into a sterile plastic universal container (10 ml) with a Pasteur pipette and aspirated gently to avoid the top 'buffy coat' layer either/or the red

cells. In order to stabilise the PRP and prevent platelet activation due to cooling, aliquots of PRP were put into siliconised glass cuvettes which were then placed in a Chronolog dual channel optical aggregometer (Coulter Electronics, Luton, Beds, UK). These aliquots were kept at 37^{o}C for 15 min (without stirring) to allow stabilisation of the platelets prior to the start of the experiments (*see Appendix A: PSC reference list with **IA Jagroop**)*.

2.3 Addition of Agonists and Fixation of Samples for PSC

At specific times after adding the agonists (as 10-50 μl volumes to achieve the final concentrations as shown in the relevant chapters methods), and stirring (using a Teflon-coated metal stir bar, at 1000 rpm), 100 μl aliquots of PRP were removed and mixed with 400μl of fixative (4% aqueous glutaraldehyde in saline). Vehicle or drugs were added (as 5-20 μl volumes), and pre-incubated for 5 min before the addition of agonists. In order to prevent platelet activation due to stirring during the pre-incubation period, the magnetic stirring mechanism within the aggregometer was switched off after the first 30 sec, and only switched back on after a 4 min interval of incubating with drug. These sampling times were determined in previous studies (*see Appendix A: PSC reference list authored by **IA Jagroop***) and also selected to represent the time when a maximal increase in MPV is observed.

2.4 Platelet Counting and Particle Size Analysis Using a Coulter ZM 34 Counter & a C-256 Channelyzer

The PRP sample which was fixed in gluteraldehyde (as described above in section 2.3) was further diluted 400 fold in Isoton II (Coulter Electronics, Luton, Beds, UK) at room temperature. A Coulter counter ZM 34 (electrical impedance method using a 70 μm diameter sampling tube orifice), coupled to a Coulter C-256 high-resolution (0.07 fl) channelyzer (Coulter Electronics, Luton, Beds, UK) was used to assess MPV in these samples **(Jagroop *et al.*, 1996; Jagroop *et al.*, 2000c; Jagroop *et al.*, 2000d; Jagroop *et al.*, 2000a; Jagroop *et al.*, 2001)** (see Appendix A PSC for full list of references authored by **IA Jagroop**).

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Figure 2.1: A ZM34 Coulter counter coupled to a C-256 Coulter channelyzer
A photograph of the 3-part machine used to assess platelet shape change (PSC). A Coulter counter ZM 34 (far right) coupled to a Coulter C-256 high-resolution (0.07 fl) channelyzer (far left) with the sampling stand (middle).

The analyser sample "window" was set between 2.67 and 19.12 fl. The counter-channelyzer system was calibrated using platelet latex particles (9 fl; Coulter Electronics). Data was accumulated to a maximum of 500 platelets in each one of the 256 channels. The MPV was the volume of the channel on each side of which 50% of the platelet population

was distributed. All MPV readings were the average of three measurements (**Jagroop *et al.*, 2002a; Jagroop *et al.*, 2001; Jagroop *et al.*, 2003a; Jagroop *et al.*, 2008**) *(see Appendix A, for full list of PSC references authored by **IA Jagroop**)*.

The platelet count was monitored to ensure that significant aggregation did not occur. The fall in platelet count was not greater than 5 % in any of the samples included in these studies. Counting of particles "channelyzed" was performed automatically by the equipment. The ZM counter is designed so that as each platelet passes through the aperture it momentarily modulates the electrical impedance between electrodes. The applied current is constant and some of the current is diverted into a preamplifier each time a platelet passes through the aperture. The amplitude of the current pulse is a function of the platelet volume (Coulter Electronics handbook). All "platelet sizing" experiments were carried out within 2 h of blood collection **(Jagroop *et al.*, 2008; Jagroop *et al.*, 1996;Jagroop *et al.*, 2000d; Jagroop *et al.*, 2000a)** *(see Appendix A, for full list of PSC references authored by **IA Jagroop**)*.

Reproducibility of MPV by this methodology has been previously evaluated, and the intra-assay and inter-assay coefficient of variation is 0.8 % and 2.9 %, respectively *(Barradas MA 1992)*.

2.5 Scanning Electron Microscopy (ScEM).

PRP was prepared (as described in section 2.2), and agonist was added (as outlined in 2.3). PRP was collected at the same time as for the corresponding PSC measurement. These samples were fixed with 950 μl glutaraldehyde (1.5%) for at least 2 h. These solutions were then filtered through a 13 mm, 0.6 μm nucleopore polycarbonate filter, by applying approximately 1 μl of the previously fixed PRP to a filter holder. This was then washed with 20 ml of distilled water. The platelets adhering to the filter, were post-fixed with osmium tetroxide for 1 h and then gently washed in distilled water. These filters were dehydrated using graded acetone (30, 50, 70, 90 and 100%), HPLC grade (Sigma, Poole, UK). The filters were then treated with tetramethylsaline (Sigma, Poole, UK), and allowed to air dry on blotting paper. The filters were then attached to aluminum stubs with double-sided tabs (TAAB Laboratory Equipment Ltd, Berkshire, UK) and coated with gold using an SC500 (EMScope) sputter coater. The stubs were examined and photographed using a Phillips 501 scanning electron microscope **(Jagroop *et al.*, 2000a).**

2.6 Transmission Electron Microscopy (TEM) for Cell Suspension

PRP was prepared (as described in section 2.2) and agonist was added (as outlined in 2.3). PRP was collected at the same time as for the corresponding PSC measurement and was fixed with 950 μl glutaraldehyde (1.5%) for at least 2 h. They were centrifuged using a 'Whatman' micro-centrifuge to form a pellet. The preparations were then washed with PBS and post-fixed using osmium tetroxide. The specimens were then further washed using distilled water and dehydrated using graded alcohol from 30% to 100%. The cells were left

in 50% alcohol/50% Lemix (Taab) epoxy-resin mixture overnight to infiltrate at 70^0C. Ultra-thin (1μm) sections were cut using a diamond knife (Diatome) on a Reichert-Jung ultra-cut microtome and collected on 3.05 mm copper grids. These sections were stained using 1% toluidene blue stain. The ultra-thin sections were viewed and photographed using a Phillips 201 transmission electron microscope (**Jagroop *et al.*, 2000a**).

2.6.1 The Resting Platelet Using TEM

Ultra-thin sections were used to visualize platelet ultra-structure (the localisation of sub-cellular components). The examination of platelets with TEM allows the observation of membrane contours as well as the recognition of the inner leaflet of membranes (see figure 1.2). The distribution of membrane or integrated macromolecules can also be visualized. Resting platelets are discoid in shape. The cytoplasm is filled with randomly dispersed organelles. A circumferential coil of microtubules lies just under the surface membrane. The dense granules are well preserved (see figure 1.2). The α-granules are clearly separated from other membranes and from the plasmalemma. The dense tubular system is ubiquitously distributed in the platelet (see figure 1.2).

The plasmalemma shows a non-random distribution of phospholipids that depend on the functional state of platelets (Zwaal and Hemker, 1982). The openings of the surface-connected system (SCS) into the plasmalemma (Behnke, 1970; White, 1970) are recognisable with ScEM and at better resolution on replicas obtained after freeze-fracturing with TEM (Reddick and Manson, 1973). The SCS in the cytoplasm of resting platelets consists of branched channels. In human platelets the dimensions of the surface of the SCS

and the plasmalemma or the membrane of alpha granules is quite comparable. Some properties of the SCS membranes resembles those of the plasmalemma: The membrane glycoproteins GPIb, GPIb/IIIa, and others are localised there (Polley *et al.,* 1981). The membrane GPIIb/IIIa complex is transported from the surface via the SCS to the α-granules of resting platelets. During stimulus-induced shape change, the SCS of resting normal human platelets disappears.

The sub-membranous cytoskeleton (SMC) regulates properties of the skeleton such as its contours and stability and is, amongst the marginal microtubules responsible for the shape of a resting platelet. Within the resting platelet there is a lose network of long acting filaments (Mani *et al.,* 2005). Filaments from actin and myosin molecules are present in a non-polymerized state and its actin is connected with the SMC (Mani *et al.,* 2005) or with the cell organelles (Linnemann *et al.,* 2008).

2.6.2 The Activated Platelet Using TEM

Using TEM it was possible to visualise the ultra-structure of resting platelets (see figure 1.2) and compare it with activated platelets (induced by ET-1 in combination with 5HT, see figure 1.3).

Ultra-thin sections of stimulus-induced, activated platelets revealed shape change and formation of spherical or oblong cells with pseudopodia. These spike-like surface extensions contained microtubules, suggesting a possible role for the tubular elements in development of the spiky extensions. This shape change during activation is accompanied by surface enlargement. Indeed it has previously been shown the apparent 'increase' in

platelet size occurs concomitantly with the pseudopodia extensions **(Jagroop *et al*., 2000a)** In the calculations of Frojmovic and Milton 1982, the increase of the surface area may be more than two-fold after shape change.

The marginal bundle of microtubules lying under the plasmalemma supports the shape of the resting platelet and is required for platelet resistance to deformation. Activated platelets undergo a rearrangement of their microtubules and this results in a change of cell deformability (White *et al.,* 1984).

The microtubules which are associated with the outer rim of the constricting contractile gel are translocated centripetally from their sub-membranous site to the centre of the cell (Escolar *et al.,* 1987; Stark *et al.,* 1991). This contractile gel comprises the mass of cell organelles and the constriction of the gel results in organelle centralisation. Platelet activation induces a disintegration of the SMC by dissociation of the GPIb-actin-binding protein complex (Fox *et al.,* 1990). An actin protein associated with regions of the plasmalemma devoid of actin filaments reversibly increases after platelet activation (Hartwig *et al.,* 1989). Activating agents effect the formation of filaments from actin and myosin molecules. Membrane glycoproteins like the fibrinogen receptor GPIIb/IIIa can connect via linker proteins. Recently, it was observed that GPIb-IX-actin-binding protein complexes, linked to actin filaments, are pulled into the cell centre by the contractile gel. Thus, platelets may exert contractile tension not only on ligands of GPIIb/IIIa (fibrinogen, fibrin) but also on vWF bound to GPIb-IX (Kovacsovics and Hartwig, 1996).

Membrane integrated glycoproteins are receptors for physiological platelet agonists (ADP, thromboxane, thrombin) adhesive proteins (fibrinogen, fibronectin, laminin,

thrombospodin, vitronectin, vWF) and for fibre-type ligands like collagen (Clemetson, 1995). The intra-cellular receptor pools from the membranes of the SCS and the α-granules can be additionally expressed at the surface following platelet activation (Woods *et al.,* 1986). During stimulus-induced shape change, the SCS of the resting normal platelets disappears (Morgenstern *et al.,* 1995). Morphometric measurements and findings of comparative studies on "normal" and "giant" platelets (reviewed in Frojmowic & Milton, 1982) or normal "grey" platelets (Morgenstern *et al.,* 1990), have already suggested that the evagination of SCS membranes causes the surface enlargement during spreading of adherent platelets (White *et al.,* 1990).

2.7 Whole Blood Platelet Aggregation

Whole blood anti-coagulated with tri-sodium citrate was collected as described in section 2.1. Aliquots of blood were put into plastic cuvettes. These were incubated at 37^0C, for a minimum of 15 min, on a whole blood Chronolog aggregometer (model 540). This stabilises the cells after blood taking. Agonists' concentrations and volumes were added and stirred using a Teflon-coated magnet spinning at 1,000 rpm (as described in the relevant sections in this thesis). Usually saline was used as a control for agonists or drug. Samples were withdrawn from the cuvettes using a pipette with a plastic tip. The time between removing the blood from the aggregometer and aspiration into the blood counter was kept to a minimum of <10 sec. This was to prevent dis-aggregation from occurring upon standing **(Jagroop *et al.,* 1996).** The timing of samples and controls are also shown in the relevant sections of this thesis. Platelet aggregation was measured with the use of an A^CT diff

Analyzer (Beckman Coulter, Inc, Fullerton, CA). Platelet aggregation was calculated on the basis of the number of 'free' platelets remaining after agonists was added (i.e. whole blood-free count, WB-FC) and was expressed as a percentage of the basal count, as below:

$$\% \text{ WB-FC} = \frac{\text{Platelet count after adding agonists}}{\text{Basal platelet count (before agonist)}} \times 100$$

The basal count was defined as the count obtained 15 sec after commencing stirring of the WB and before addition of any agonists. All WB aggregation studies were completed within 2 h of sample collection. Blood was kept at 37^{o}C throughout aggregation experiments since platelet function may be influenced by cooling (Mikhailidis, *et al.,* 1983).

2.8 Intra-platelet Serotonin

Serotonin, or 5-hydroxytryptamine is a molecule that is found in large quanti-ties in the enterochromaffin cells of the gastrointestinal mucosa, in platelets, and in the serotoninergic neurons of the central nervous system. 5HT is synthesized via hydroxylation of tryptophan and subsequent decarboxylation.

5HT is a potent vasoconstrictor and neurotransmitter, and has multiple other complex effects. It has been implicated in the regulation of neuroendocrine function, body temperature and blood pressure. The enzyme immunoassay of 5HT (Immunotech, Beckman Coulter, High Wycombe, Bucks, UK) is based on the competition between modified 5HT in the sample and the 5HT-enzyme conjugate, used as a tracer, for binding to the antibody

coated onto microtitre plates (Fawcett & **Jagroop** *et al.,* 1998; Frampton & **Jagroop** *et al.,* 2006; **Jagroop *et al.,* 2004).**

Acylated 5HT in the sample or standards and 5HT-acetylcholinesterase conjugate were added to the microtitre wells where they compete for a limited number of anti-body-binding sites. After incubation, the contents of the wells were aspirated and the wells were washed to remove excess tracer. Bound enzymatic activity was measured by the addition of a chromogenic substrate. The intensity of the colour was a function of the concentration of 5HT in the sample. The concentration was determined from a standard curve established with the standards provided in the kit (Fawcett & **Jagroop** *et al.,* 1998; Frampton & **Jagroop** *et al.,* 2006; **Jagroop *et al.,* 2004).**

2.9 Soluble P-selectin

P-Selectin is a cell surface glycoprotein that plays a critical role in the migration of lymphocytes into tissues. It is found in a pre-formed state in the Weibel-Palade bodies of endothelial cells and in the alpha granules of platelets. P-Selectin is mobilised to the cell surface within minutes in response to a variety of inflammatory or thrombogenic agents (Antoniades C *et al*., 2010).

Studies indicate that P-Selectin is involved in the adhesion of myeloid cells, along with B cells and a subset of T cells, to activated endothelium (Mestas *et al.,* 2008; McGregor *et al.,* 2006). P-Selectin is also involved in the adhesion of platelets to monocytes and neutrophils, playing a central role in neutrophil accumulation within thrombi. P-Selectin is found in the plasma of normal individuals at 'ng/ml' concentrations. Circulating P-

Selectin appears to be slightly smaller than native P-Selectin. An alternatively spliced mRNA encoding a form of human P-Selectin lacking the transmembrane anchoring domain has been reported for both megakaryocytes and endothelial cells, and evidence suggests that the majority of circulating soluble P-Selectin arises in this manner (Mestas *et al.,* 2008). A number of studies have reported that levels of soluble P-Selectin in biological fluids may be elevated in subjects with a variety of pathological conditions (Polek *et al.,* 2009).

Only plasma is acceptable. Plasma was collect on ice using EDTA or heparin as anti-coagulant. Anit-coagulated blood was centrifuge at 2-8°C for 15 min at 1000 x g within 30 min of collection. An additional centrifugation step of the separated plasma at 10000 x g for 10 min at 2-8°C was recommended for complete platelet removal. The assay was either done immediately or aliquoted and stored at or below -20°C. Freeze-thaw cycles were avoided.

The assay was done with the use of the human soluble sP-selectin quantitative sandwich immunoassay kit (R&D Systems Europe, Oxon, UK). A monoclonal anti-body specific for sP-Selectin was pre-coated onto a microplate. Standards, samples and controls were pipetted into the wells, together with a polyclonal anti-body specific for sP-Selectin, which was conjugated to horseradish peroxidase. After removal of unbound conjugated anti-body, a substrate was added and colour was developed in proportion to the sP-Selectin concentration.

2.10 Platelet Derived Growth Factor

There are 3 isoforms of PDGF-AA, -AB, and –BB, which are expressed in many cell types. Plasma PDGF-AB is measured as this isoform is more likely to represent a human platelet source. PDGF acts as a growth factor in many cell types and it may also influence platelet activity.

This human PDGF-AB assay employs the quantitative sandwich enzyme immunoassay technique (R&D Systems Europe, Oxon, UK). A monoclonal antibody specific for PDGF-BB was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any PDGF-AB present was bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal specific for PDGF-AA was added to the wells. Following a wash to remove any unbound anti-body-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of PDGF-AB bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

2.11 Statistics in Brief

Randomisation : Randomisation is important for the design of good experiments as it substantially reduces the chance of bias which could arise if subjects/samples on one treatment are in some way different from those on another treatment. Physical randomisation is fairly easy to do in 'small' experiments. In the clopidogrel/aspirin study (Chapter 3), a random number allocation procedure was applied by Ealing hospital Pharmacy.

Power calculations*:* Power calculations are important to determine the appropriate sample size for an experiment. However, power calculations are difficult to use for pilot studies because the effect of the intervention is not known. In this context, in Chapter 3, the effect of aspirin and/or clopidogrel on PSC (as measured by MPV) was not known prior to the study. Hence, a power estimation was not carried out.

The power calculation to estimate sample size depends on a mathematical relationship between 6 variables, these include:

- *Variability of the material* i.e. an estimate of the SD of the experimental subjects is necessary and comes from a previous study.
- *Effect of size of clinical or biological importance* i.e. the ability to specify the minimum effect size likely to be of interest.
- *Significance level* i.e. this is usually 0.05 but power will be higher if it is set at 0.1 rather than 0.05.
- *Power* i.e. the probability to detect the specified effect that is considered significant, at the designated level of significance.
- *Sidedness of the test* i.e. a two-sided test is usually used except where there is a good biological reason why the treatment effect can only go in one direction.
- *Sample size* i.e. the aim of the power analysis is usually to determine sample size of the study.

Multiple Comparisons: The term "comparisons" in multiple comparisons typically refers to comparisons of 2 groups, such as a treatment group and a control group. "Multiple comparisons" arise when a statistical analysis encompasses a number of formal comparisons. A useful guide is to use a *Bonferroni* correction which states simply that if one is testing *n* independent hypothesis, one should use a significant level of 0.05/*n*. However, other methods have been described (Kusuoka *et al.,* 2002). For example in this thesis where if 2, 3 or 4 pairs are compared, the level of significance is considered as 0.049, 0.047 or 0.046 respectfully for each of the pairs (Kusuoka *et al.,* 2002).

CHAPTER 3

The Effect of Clopidogrel, Aspirin or Both Antiplatelet Drugs on Platelet Function in Patients with Peripheral Arterial Disease

3.1 (i) AIMS, BACKGROUND AND HYPOTHESIS

Aims: The aim of this study was to investigate if clopidogrel plus aspirin combination therapy provides more potent platelet inhibition than monotherapy with clopidogrel or aspirin in patients with PAD. I also compared the effect of each monotherapy. Furthermore, I assessed if these antiplatelet drugs inhibit early platelet activation (i.e. PSC).

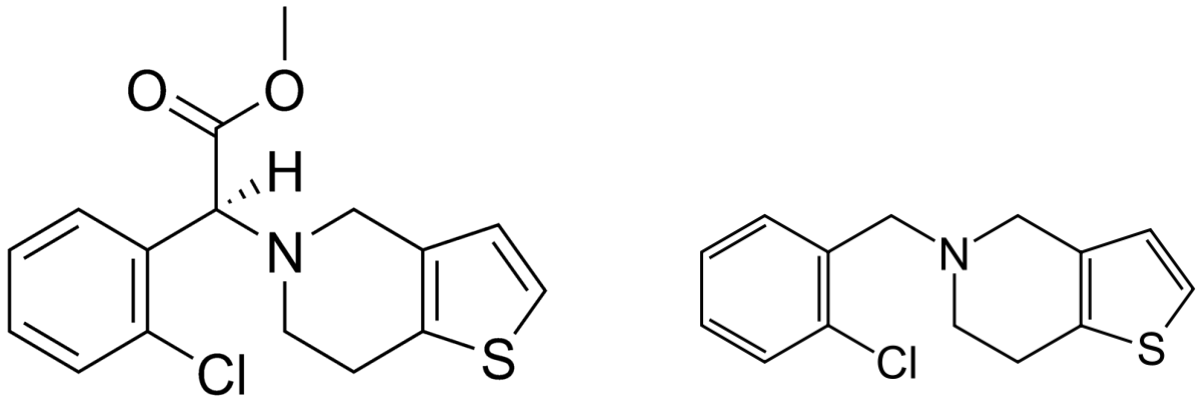
Background: PAD patients are often prescribed aspirin. However, aspirin only affects one pathway of platelet activation (preventing the conversion of arachidonic acid to TXA_2, a potent vasoconstrictor and platelet agonist). PAD patients may experience a resistance to aspirin. Thus, the thienopyridines (e.g. clopidogrel) may provide and effective alternative to aspirin. The thienopyridines were developed as other antiplatelet drugs that target other pivotal pathways such as those mediated by ADP. Clopidogrel (a thienopyridine) inhibits platelet activation by blocking the ADP $P2Y_{12}$ purinergic receptor. The rational behind combination therapy is that aspirin and clopidogrel act via different mechanisms.

Previous studies have shown that clopidogrel can affect platelet aggregation. But no other investigators have yet demonstrated any effectiveness of this drug on PSC using a high-resolution channelyzer method. PSC is important because it is an early phase of platelet activation that precedes platelet aggregation.

Hypothesis: My hypothesis was that clopidogrel would be superior to aspirin in terms of inhibiting platelet activity (including PSC). Furthermore, the combination of these 2 drugs would be superior to monotherapy.

3.1 (ii) INTRODUCTION

Clopidogrel is a theinopyridines and can inhibit ADP-induced inhibition of adenylate cyclase, prevent the ADP-induced inhibition of cytoskeletal associated protein vasodilator-stimulated phosphoprotein (VASP) phosphorylation and prevent the association of labeled G proteins with the platelet membrane (Duerschmied *et al.,* 2010; Quinn *et al.,* 1999). Other drugs belonging to this group include Prasugrel (Effient) and ticlopidine (Ticlid) (Engelen *et al.,* 2010). Ticlopidine was the first of this new class of theinopyridines.



Clopidogrel Ticlopidine

Figure 3.1: Molecular structures of clopidogrel and ticlopidine

Ticlopidine gained FDA approval for clinical use in the early 1990's. Clopidogrel emerged after as a promising compound structurally similar to ticlopidine. However, the difference is that unlike ticlopidine, clopidogrel has an additional carboxymethyl side group (Porto *et al.,* 2009; Preobrazhenskii *et al.,* 2009; Jacobson, 2004)

Clopidogrel is a pro-drug and needs to be converted *in vivo* to active metabolites by the hepatic cytochrome P-450 (Weksler *et al.,* 1977; Duerschmied *et al.,* 2010). Their active metabolite then binds to the $P2Y_{12}$ receptor and forms a disulphide bond with a cysteine residue of the receptor, thereby irreversibly inhibiting it (Jacobson, 2004). Clopidogrel is absorbed rapidly via the oral route (Marczewski *et al.,* 2010). Metabolism is so extensive that clopidogrel is difficult to detect in the circulation; the major circulating compound is the inactive carboxylic acid derivative SR26334 that reaches peak plasma concentration within 1 h of ingestion (Easton, 1999).

Clopidogrel has been approved by the FDA for reduction of thrombotic events in recent MI, stroke, or established PAD, and for patients with non-ST-elevation acute coronary syndromes including percutaneous coronary interventions (Easton, 1999; Jacobson, 2004).

Clopidogrel is an effective inhibitor of platelet activity **(Jagroop** *et al.,* 2007**).** Clopidogrel was more clinically effective when compared with aspirin in a very large trial (n = 19,185; CAPRIE) that involved patients with established vascular disease (1996, *no authors listed*). In other studies (e.g. CURE), aspirin and clopidogrel combination therapy was more effective than aspirin alone in patients with acute coronary syndrome (Mehta *et al.,* 2000). The rationale behind combination therapy is that aspirin and clopidogrel act by different mechanisms. Aspirin inhibits the synthesis of thromboxane A_2 (TXA_2), a promoter of platelet aggregation (Drouet *et al.,* 2010). In contrast, clopidogrel acts at the level of the platelet ADP purinergic ($P2Y_{12}$) receptors (Marczewski *et al.,* 2010; Mikhailidis *et al.,* 1998b). ADP acts on the $P2Y_{12}$ receptor to enhance aggregation and on the $P2Y_1$ receptors

to PSC and aggregation (Kim *et al.,* 2011). PSC is an early phase of platelet activation that precedes aggregation and is essentially aspirin resistant *in vivo* **(Jagroop *et al.,*** 2000d; **Jagroop** *et al.,* 2000a; **Jagroop** *et al.,*2001; **Jagroop** *et al.,* 2003a) *(see Appendix A, for full list of PSC references authored by **IA Jagroop**).*

Aspirin has been the anti-platelet drug of choice for many decades but it only affects a single pathway involved in platelet activation. It prevents the conversion of AA to TXA_2, a potent vasoconstrictor and platelet agonist, by irreversibly inactivating the COX activity of PG H-synthase-1 and PG H-synthase-2 (also referred as COX-1 and COX-2) (Jneid, 2010; Mackenzie *et al.,* 2010; Sanchez-Ross *et al.,* 2010).

Therefore, thienopyridines (e.g. ticlopidine and clopidogrel) which inhibit platelet activation through the blockade of the ADP pathway may provide an effective alternative to aspirin therapy (Gachet, 2001; Lubbe, 2002).

ADP plays a major role in platelet activation. It is present in high concentrations in platelet dense granules. ADP is released when platelets are stimulated by other agonists (e.g. thrombin or collagen) or even ADP itself high to further induce platelet aggregation. ADP, even in low concentrations, when combined with other agonists (e.g. adrenaline or 5HT) can have a synergistic effect on platelets (Gachet, 2001).

PAD is a common condition which involves the arteries of the lower limbs. PAD patients tend to have hyperactive platelets that may be resistant to aspirin (Matsagas *et al.,* 2003; Robless *et al.* 2001). Therefore, it is relevant to assess the effect of other platelet inhibitors, like clopidogrel, which acts via a different mechanism.

Identifying effective anti-platelet therapy in patients with PAD is potentially very important because this condition is associated with an increased risk of myocardial infarction (MI) and stroke (Barradas *et al.,* 1994b; Robless *et al.,* 2001; Matsagas *et al.,* 2002).

We have previously shown that a loading dose of clopidogrel (300 mg) inhibited ADP- and 5HT-induced PSC and aggregation in patients with PAD (Matsagas & **Jagroop** *et al.,* 2003). However, it is not know if the daily dose of clopidogrel exerts similar effects in patients with PAD. In addition, the effect of combination therapy (clopidogrel plus aspirin) has not been previously assessed with a sensitive PSC methodology. In the present study, these questions were addressed by comparing patients with PAD on clopidogrel or aspirin or on combination therapy (clopidogrel plus aspirin).

3.2 METHODS

3.2.1 Selection of Patients and Collection of Blood

This study was approved by the Local Ethics Committee of the Ealing Hospital NHS Trust, London, UK and the Royal Free Hospital NHS Trust, London, UK. Written information was given to each participating patient who signed a consent form.

Twenty patients, 14 men and 6 women, (mean age 69.8 years, range 58-77 years) with intermittent claudication took part in the study. All patients attended the vascular surgery clinic at Ealing Hospital. PAD was previously diagnosed by history and clinical examination and all patients had an Ankle-Brachial Pressure Index (ABPI) < 0.80 (median 0.62; range 0.4-0.75) at rest. All patients had a standard treadmill exercise test (3.5 km/h,

10^{o} incline), which recorded a significant post-exercise drop (>20%) in the ABPI. All patients were in a metabolically and clinically stable condition, with no history of recent (6 months) cardiac events, strokes, transient ischaemic attacks or changes in their claudication distance. These patients had not undergone any recent (6 months) surgery, angiography or angioplasty.

Seven of the patients were never treated with any anti-platelet agent in the past, 11 were on aspirin 75 mg per day, while two of them were receiving the combination of aspirin 75 mg per day and dipyridamole 200 mg twice per day. These last 13 patients had to discontinue their anti-platelet therapy for at least 10 days before taking part in the study. None of the patients had renal impairment. A total of 6 patients had type 2 (non-insulin dependent) diabetes mellitus, (NIDDM) 12 were being treated for hypertension and 8 for ischaemic heart disease (IHD).

Randomisation is essential for any experiment as it minimizes the chance of a biased result. Patients were randomised for treatment with either aspirin or clopidogrel or both drugs together. Randomization was carried out by the Pharmacy Dept. at Ealing Hospital according to the usual procedure (random number allocation). Myself and the patients were blinded to this information, until the end of the study. Placebo was given with either aspirin or clopidogrel, so that 2 tables were always given to the patients. In the phase of combination therapy (aspirin plus clopidogrel) both tablets consisted of active treatment (i.e. one tablet was aspirin and one tablet was clopidogrel). This was known to the investigators and patients. In other words the second phase of this study was not blinded.

There were no differences in sex or DM distribution between the two groups. All patients were transported to the Department of Clinical Biochemistry at the Royal Free Hospital and a baseline citrated venous blood sample was collected after resting for 15 min. Group A patients were given clopidogrel (Plavix, Sanofi-Synthelabo, Paris, France) 75 mg/day and placebo for 8 days.

In group B, patients were given aspirin (75 mg/day) and placebo for 8 days. On day 8, blood samples were collected in a similar way from all patients, 4 h ± 30 min after the last medication had been taken. After that, all patients in both groups took the combination of the two drugs (aspirin 75 mg/day plus clopidogrel 75 mg/day) for 8 more days. On the last day, 4 h ± 30 min after both medications were taken, a blood-sample was collected.

3.2.2 Platelet Aggregation

Platelet aggregation was measured in citrated (3.8 %) whole blood (WB) using an MD18 Coulter counter (Coulter Electronics, Luton Beds, UK). Agonist-driven aggregation was assessed (*see Appendix B: Platelet aggregation reference list with **IA Jagroop**)* by counting the number of free platelets (as the % free platelet count, FPC), remaining in the stirred sample (at 37^{o}C), after adding adenosine diphosphate (ADP; 5-10 μmol/l) or 5HT (5.0 μmol/l). Spontaneous platelet aggregation (SPA) was followed up for 15 min at specific sampling time points (0, 3, 6 and 15 min). The rationale was to assess platelet aggregation that has been reported as enhanced in patients with PAD (Matsagas, *et al.,* 2002; Mikhailidis, *et al.,* 1998; Robless, *et al.,* 2003). In addition, SPA is substantially ADP-

dependent and aspirin resistant (Matsagas, *et al.,*. 2002; Robless, 2003) SPA (in PRP) has been shown to be a predictor of the risk of a vascular event (Thaulow, *et al.,*. 991)

3.2.3 Platelet Shape Change

We used a high-resolution (0.07 fl) C256 channelyzer (electrical impedance method with a 70μm diameter sampling tube orifice) coupled to a ZM34 Coulter counter (Coulter Electronics, Luton Beds, UK) to determine MPV of human platelets (as described in General Methods chapter 2). PSC is an early phase of platelet activation that precedes aggregation (*see Appendix A: PSC reference list with **IA Jagroop**)*. PSC may be associated with platelet adhesion to the vascular wall (Rosenfeld *et al.,* 1997). Citrated (3.8 %) PRP was incubated at 37^{o}C for 15 min, prior to the start of the experiment. Agonists (5HT 0.5 μmol/l, ADP 0.4-0.8 μmol/l) were added to PRP and stirred at 1000 rpm. These agonists concentrations and sampling times were determined in previous studies; they generate the most reproducible results. The percentage increase in MPV was calculated by using the saline control as baseline, and comparing this value with agonist- induced MPV (*see Appendix A: PSC reference list authored by **IA Jagroop**)*.

3.2.4 Intra-platelet 5HT Measurement

3.2.4.1 Collection and Processing of Specimen

Blood was collected in cold (2-8^{0}C) polystyrene tubes (as described in General Methods Chapter 2), with a specific solution (Gow *et al.,* 1988) that inhibits the enzymatic

oxidation and cellular uptake of 5HT (9 parts blood to 1 part solution) which contained the following dissolved in physiological saline (Fawcett and **Jagroop** *et al.,* 1998; Frampton and **Jagroop** *et al.,* 2006; **Jagroop** *et al.,* 2004).

EDTA 50 mM	PGE_1 500 nM
clorgyline 100 μM	chlorimipramine 100 μM
pargyline 100 μM	

The tubes were mixed gently by inversion and centrifuged immediately in a cold centrifuge (4^{o}C) at x120g, for 15 min, to obtain PRP. Each sample was analysed for a platelet count using the Coulter MD18. This count was recorded, as it is necessary to calculate the platelet 5HT concentration later in the assay. 1 ml aliquots of PRP were put into cold Eppendorf tubes and microcentrifuged (x10,000g) for 2 min to prepare a platelet pellet. The platelet poor palette (PPP) was carefully collected, using a Pasteur pipette and stored at -20^{o}C. The remaining pellet was washed with Isoton II, and stored at -20^{o}C. Each Eppendorf tube containing the pellet was resuspended in 1 ml physiological saline (0.9% w/v) and a platelet lysate prepared by freeze thawing 3 times followed by ultrasonication (3 x 10 sec at an amplitude of 18 microns) using an MSE-Soniprep probe-sonicator (Fawcett and **Jagroop** *et al.,* 1998; Frampton and **Jagroop** *et al.,* 2006; **Jagroop** *et al.,* 2004). In order to ensure full disruption of the platelets, the platelet populations were counted and sized before and after sonication using a Coulter counter coupled to a channelyzer (see Chapter 2, section 2.4). If the platelet count following this procedure was reduced to <5% of the original, the platelet volume was disregarded since it became unmeasurable.

3.2.4.2 Intra-platelet 5HT Enzyme Immunoassay

Intra-platelet 5HT concentrations were determined using an enzyme immunoassay kit (Immunotech-Beckman Coulter). The principle of this assay is based on the competition between modified 5HT in the sample and the 5HT-enzyme conjugate, used as a tracer, for binding to the anti-body coated onto microtitre plates (Fawcett and **Jagroop** *et al.,* 1998; Frampton and **Jagroop** *et al.,* 2006; **Jagroop** *et al.,* 2004).

3.2.5 Soluble P-Selectin (sP-Selectin) Immunoassay

See General Methods (Chapter 2) (**Jagroop** *et al.,* 2004; Mohan and **Jagroop** *et al.,* 2008).

3.2.6 Platelet-derived growth factor-AB (PDGF) immunoassay

See General Methods (Chapter 2) (**Jagroop** *et al.,* 2004).

3.2.7 Statistical Analysis

Results are presented as mean ± SD in tables 3.1-3.5 where comparisons are made using a paired t-test (two-tailed). In tables 3.3 and 3.4 results are expressed as median and range the P values were derived by non-parametric analysis (paired Wilcoxon tests, two tailed).

3.3 RESULTS

3.3.1 Platelet Aggregation

3.3.1.1 Spontaneous Platelet Aggregation

There was no significant change in SPA after taking clopidogrel or aspirin alone for 8 days (table 3.1). However, after combination therapy (clopidogrel plus aspirin) SPA was significantly inhibited (6 min; P = 0.02 and 15 min; P = 0.001) in both groups, i.e. whether the patients were initially assigned to aspirin or clopidogrel; n = 10 for each group and n = 20, when grouped together (see table 3.3).

Table 3.1: Spontaneous Platelet Aggregation

	3 min	6 min	15 min
Clopidogrel V1 vs V2 **P Value**	87 ± 9 vs 88 ± 11 **NS**	73 ± 9 vs 78 ± 12 **NS**	58 ± 13 vs 66 ± 18 **NS**
Aspirin V1 vs V2 **P Value**	84 ± 15 vs 84 ± 11 **NS**	74 ± 20 vs 78 ± 20 **NS**	63 ± 17 vs 58 ± 24 **NS**
Clopidogrel V2 vs Aspirin V2 **P Value**	88 ± 11 vs 84 ± 11 **NS**	78 ± 12 vs 78 ± 20 **NS**	66 ± 18 vs 58 ± 24 **NS**

Spontaneous platelet aggregation (SPA) is measured as % free platelet count. Results are expressed as mean ± SD (n = 10 for each drug), using a paired t-test. Comparing visit 1 (V1, baseline) with visit 2 (V2, after taking clopidogrel or aspirin for 8 days). Also comparing taking clopidogrel or aspirin after 8 days (V2 on clopidogrel vs V2 on aspirin). Results are expressed as mean ± SD (n = 10 for each drug), using a paired t-test.

3.3.1.2 ADP-induced Aggregation

After taking clopidogrel for 8 days there was a significant decrease (P = 0.001 - 0.0003 at 3, 6 and 15 min) in ADP (5 μmol/l)-induced aggregation (table 3.2). In contrast, no significant inhibition of aggregation was observed after taking aspirin for the same time. This effect appeared more obvious when those patients who took aspirin only at visit 2 (after 8 days), were compared with those who took clopidogrel only at the same visit. ADP (5 μmol/l)-induced aggregation was significantly decreased for the group on clopidogrel at all the time points measured (e.g. P = 0.003 at 3 min; table 3.2). This trend was repeated with a concentration of 10 μmol/l ADP-induced aggregation (table 3.3b).

Taking combination therapy (clopidogrel plus aspirin) after 8 days showed that ADP-induced aggregation (at both concentrations of 5 μmol/l and 10 μmol/l), was significantly (P <0.03 to P = 0.0004) inhibited in both patient groups (i.e. whether the patients were assigned to aspirin or clopidogrel; n= 10 for each group (see table 3.3b).

When the patients were grouped together (n = 20), taking combination therapy (clopidogrel plus aspirin) after 8 days showed that ADP-induced aggregation (at both concentrations of 5 μmol/l and 10 μmol/l), was significantly (e.g. P ≤ 0.0001 - 0.0003) inhibited (see table 3.3a)

Table 3.2: ADP (5 μmol/l)-induced Platelet Aggregation

	3 min	6 min	15 min
Clopidogrel V1 vs V2 **P Value**	46 ± 22 vs 83 ± 13 **0.0001**	42 ± 11 vs 77 ± 15 **0.0003**	43 ± 12 vs 72 ± 14 **0.001**
Aspirin V1 vs V2 **P Value**	43 ± 24 vs 56 ± 21 **NS**	45 ± 20 vs 57 ± 21 **NS**	43 ± 16 vs 48 ± 18 **NS**
Clopidogrel V2 vs Aspirin V2 **P Value**	83 ± 13 vs 56 ± 21 **0.003**	77 ± 15 vs 57 ± 21 **0.03**	72 ± 14 vs 48 ± 18 **0.005**

Platelet aggregation (measured as % free platelet count).
Comparing visit 1 (V1, baseline) with visit 2 (V2, after taking clopidogrel or aspirin for 8 days). Also comparing taking clopidogrel or aspirin after 8 days (V2 on clopidogrel vs V2 on aspirin). Results are expressed as mean ± SD (n = 10 for each drug), using a paired t-test.

Table 3.3a: Spontaneous and Agonist-induced Platelet Aggregation (Effect of Clopidogrel plus aspirin combination, n=20)

	Baseline,V1 vs Clopidogrel plus Aspirin combination,V3	
SPA		
3 min	85 ± 12 vs 87 ± 9	P = **NS**
6 min	73 ± 15 vs 83 ± 10	**P = 0.02**
15 min	60 ±15 vs 77 ± 12	**P = 0.001**
ADP 5 µmol/l		
3 min	44 ± 23 vs 76 ± 17	**P = 0.0003**
6 min	43 ± 16 vs 76 ± 17	**P = 0.0001**
15 min	43 ± 13 vs 73 ± 18	**P = 0.0001**
ADP 10 µmol/l		
3 min	31 ± 27 vs 70 ± 21	**P ≤ 0.0001**
6 min	35 ± 19 vs 70 ± 16	**P ≤ 0.0001**
15 min	34 ± 20 vs 68 ± 16	**P ≤ 0.0001**
5HT 5 µmol/l		
6 min	65 ± 17 vs 72 ± 21	**P = NS**
15 min	50 ± 19 vs 64 ± 15	**P = NS**

Baseline (V1) is compared with taking a combination of clopidogrel plus aspirin (V3).Results are expressed as mean ± SD (measured as % free platelet count, n=20), using a paired t-test.

Table 3.3b: Spontaneous and Agonist-induced Platelet Aggregation (Effect of Clopidogrel plus aspirin combination, n=10)

	V1 v V3 (group initially assigned to clopidogrel)		V1 v V3 (group initially assigned to aspirin)	
SPA				
3 min	87 ± 9 v 89 ± 12	**P=NS**	84 ± 15 v 85 ± 6	**P=NS**
6 min	73 ± 9 v 88 ± 12	**P = 0.006**	66 ± 19 v 86 ± 8	**P=0.03**
15 min	58 ± 13 v 79 ± 11	**P=0.002**	56 ± 17 v 77 ± 11	**P=0.01**
ADP (5µM)				
3 min	46 ± 22 v 78 ± 14	**P=0.003**	43 ± 24 v 72 ± 20	**P=0.014**
6 min	42 ± 10 v 77 ± 14	**P=0.0004**	45 ± 20 v 73 ± 23	**P=0.004**
15 min	43 ± 11 v 73 ± 15	**P=0.0004**	43 ± 16 v 64 ± 24	**P=0.001**
ADP (10µM)				
3 min	37 ± 30 v 80 ± 8	**P=0.01**	25 ± 23v 58 ± 25	**P=<0.02**
6 min	37 ± 19 v 75 ± 12	**P=0.001**	33 ± 21 v 55 ± 18	**P=<0.03**
15 min	34 ± 9 v 70 ± 13	**P=0.002**	30 ± 15 v 58 ± 20	**P=0.001**
5HT (5µM)				
6 min	68 ± 17 v 79 ± 13	**P=NS**	63 ± 18 v 69 ± 15	**P=NS**
15 min	56 ± 16 v 74 ± 16	**P=<0.02**	45 ± 20 v 63 ± 14	**P=0.03**

SPA, ADP- and 5HT-induced platelet aggregation (measured as % free platelet count) after taking aspirin plus clopidogrel (V3) versus no treatment in those who were initially assigned clopidogrel mono-therapy. The same comparison was also carried out in those initially assigned aspirin mono-therapy. Results are expressed as mean ± SD (n = 10). The *P* value was derived using a paired t-test.
V1, baseline sample (pre-treatment); V3, treatment with aspirin plus clopidogrel.

Table 3.4: ADP (10 µmol/l)-induced Aggregation

	3 min	6 min	15 min
Clopidogrel V1 vs V2 **P Value**	37 ± 30 vs 71 ± 9 **0.004**	37 ± 19 vs 68 ± 9 **0.005**	34 ± 9 vs 66 ± 13 **0.0006**
Aspirin V1 vs V2 **P Value**	25 ± 22 vs 33 ± 24 **NS**	33 ± 21 vs 34 ± 22 **NS**	34 ± 22 vs 38 ± 20 **NS**
Clopidogrel V2 vs Aspirin V2 **P Value**	71 ± 9 vs 33 ± 24 **0.0002**	68 ± 9 vs 34 ± 22 **0.014**	66 ± 13 vs 38 ± 20 **0.005**

Platelet aggregation (measured as % free platelet count).
Comparing visit 1 (V1, baseline) with visit 2 (V2, after taking clopidogrel or aspirin for 8 days). Also comparing taking clopidogrel or aspirin after 8 days (V2 on clopidogrel vs V2 on aspirin). Results are expressed as mean ± SD (n = 10 for each drug), using a paired t-test.

3.3.1.3 5HT-induced Aggregation

5HT-induced aggregation was not significantly inhibited by either clopidogrel or aspirin (table 3.5) on their own. The combination therapy, clopidogrel together with aspirin, also appeared to demonstrate no significant effect on 5HT-induced platelet aggregation, when both patients groups (i.e. those initially assigned aspirin, n=10, plus those initially assigned clopidogrel, n=10) were pooled, before they were given combination therapy, (n=20) (see table 3.3a). However, when the 2 patient groups were considered individually (see table 3.3b), after taking combination therapy for 8 days. 5HT-induced aggregation was significantly ($P=0.03$ to $P<0.02$) inhibited at 15 min, in both patient groups (i.e. whether the patients were initially assigned to aspirin or clopidogrel; n=10 for each group, before being given the combination therapy) (see table 3.3.b).

Table 3.5: 5HT (5 µmol/l)-induced Aggregation

	6 min	15 min
Clopidogrel V1 vs V2 **P Value**	68 ± 17 vs 79 ± 10 **NS**	56 ± 16 vs 67 ± 15 **NS**
Aspirin V1 vs V2 **P Value**	63 ± 18 vs 68 ± 18 **NS**	45 ± 20 vs 55 ± 17 **NS**
Clopidogrel V2 vs Aspirin V2 **P Value**	79 ± 10 vs 68 ± 18 **NS**	67 ± 15 vs 55 ± 17 **NS**

Platelet aggregation (measured as % free platelet count).
Comparing visit 1 (V1, baseline) with visit 2 (V2, after taking clopidogrel or aspirin for 8 days). Also comparing taking clopidogrel or aspirin after 8 days (V2 on clopidogrel vs V2 on aspirin). Results are expressed as mean ± SD (n = 10 for each drug), using a paired t-test.

3.3.2 Platelet Shape Change Results

3.3.2.1 ADP-induced PSC

PSC induced by ADP (0.8 µM, at 30 and 60 sec) was more significantly inhibited (P = 0.004) by clopidogrel alone than by aspirin alone (P = 0.01, table 3.6). With combination therapy (table 3.7), ADP-induced PSC was even more significantly inhibited (P = 0.0002), than by either drug on its own.

3.3.2.2 5HT-induced PSC

5HT-induced PSC was significantly inhibited by clopidogrel as well as by aspirin (table 3.6), but only at the 30 sec time point. With combination therapy (table 3.7), 5HT-induced PSC at both time points (30 and 60 sec) was also significantly inhibited (P = 0.0006 and P = 0.001, respectively).

Table 3.6: Agonist-induced Platelet Shape Change Pre- and Post-aspirin or Clopidogrel

	Base (V1) vs Clop (V2)	**P value**	Base (V1) vs ASA (V2)	**P value**
ADP 0.4 μM				
30 s	4.9 (3.3 -11.0) vs 2.4 (0 -9.3)	**P = 0.01**	6.7 (3.3 - 21.8) vs 5.2 (1.0 - 13.9)	**P = NS**
60s	6.1 (3.4 - 10.2) vs 2.3 (0- 9.9)	**P = 0.01**	7.1 (3.8 - 16.0) vs 5.7 (2.0 - 15.2)	**P = NS**
ADP 0.8 μM				
30 s	10.1 (3.5 - 18.6) vs 6.4 (0.3 - 9.7)	**P = 0.004**	10.3 (6.7 - 20.9) vs 7.5 (2.1 - 11.8)	**P = 0.01**
60 s	10.5 (2.9 - 14.5) vs 5.6 (0 - 14.2)	**P = 0.004**	10.1 (7.5 - 23.9) vs 7.5 (3.8 - 12.01)	**P = 0.01**
5HT 0.5 μM				
30 s	8.3 (4.9 - 14.4) vs 6.6 (2.2 - 8.7)	**P = 0.01**	11.7 (5.0 - 18.4) vs 6.2 (0.5 - 10.8)	**P = 0.03**
60 s	8.0 (6.0 - 14.6) vs 5.8 (1.8 - 12.0)	**P = NS**	9.9 (5.31 - 14.5) vs 6.2 (1.9 - 11.8)	**P = NS**

Platelet shape change (PSC, using % increase in median platelet volume-MPV).
Comparing Baseline (V1) with either clopidogrel or aspirin after 8 days (V2).
Results expressed as median using non-parametric Wilcoxon matched pairs (n= 10 for each drug).

Table 3.7: Agonist-induced Platelet Shape Change Pre- and Post-Combination Treatment

	Base (V1) Vs Clopidogrel + Aspirin combination (V3)	**P**
ADP 0.4 μM 30 s 60s	5.5 (3.3 - 21.8) vs 2.3 (0 - 12.3) 6.8 (3.4 - 16.0) vs 1.7 (0 - 8.8)	**P = 0.001** **P = 0.0005**
ADP 0.8 μM 30 s 60 s	10.2 (3.5 - 20.9) vs 5.3 (0 - 16.2) 10.4 (2.9 - 23.9) vs 3.3 (0 - 18.0)	**P = 0.0002** **P = 0.001**
5HT 0.5 μM 30 s 60 s	8.7 (4.9 - 18.4) vs 4.5 (0 -12.0) 8.4 (5.3 - 14.6) Vs 3.8 (0 - 12)	**P = 0.0006** **P = 0.001**

Platelet shape change (PSC, using % increase in median platelet volume-MPV).
Comparing baseline (V1) with clopidogrel plus aspirin combination (V3).
Results expressed as median using non-parametric Wilcoxon matched pairs (n=20).

3.3.3 Intra-platelet 5HT Enzyme Immunoassay

Intra-platelet 5HT concentration showed no significant difference when comparing V1 with V3; from 1.0 (0.6 – 19.5) to 0.9 (0.2 – 5.5) nM/10^9 platelets.

3.3.4 Soluble P-Selectin (sP-Selectin) Immunoassay

A comparison was made with baseline values (V1) when patients were not on treatment, to when they were on combination therapy (V3). There was a significant decrease *(P = 0.04)* in sP-selectin (from 32 ± 24 to 25 ± 17 ng/ml. The comparison of baseline (V1) values with either drug alone (V2), was not considered as the numbers would have been too small (n=10), and therefore, weakening the strength of the stastical test.

3.3.5 Platelet-Derived Growth Factor-AB Immunoassay

There was no significant difference in plasma PDGF-AB when comparing baseline (V1) with the end of the study values (V3); from 92 (0 – 694) to 80 (2 – 1314) pg/ml. The range of the assay is wide ranging from non-detectable to 1143 pg/ml. Baseline values (V1) were again not compared with V2 for the reasons outlined (in 3.3.4) above.

3.4 SIDE EFFECTS

None of the patients developed any adverse events during the study. All the patients were followed up for an additional 2 weeks, but no side effects were reported. Of the 20 patients, 13 went back to their original medication, while the 7 who were previously not on anti-platelet therapy started taking aspirin (75 mg/day).

3.5 DISCUSSION

The results from this study indicate that combination therapy (clopidogrel plus aspirin) provides more potent platelet inhibition than monotherapy (clopidogrel or aspirin) in patients with PAD.

ADP-induced platelet aggregation was significantly inhibited by clopidogrel but not by aspirin. Thus, as expected, there is some degree of ADP-specificity for clopidogrel compared to aspirin (Gachet, 2001b; Lubbe *et al.,* 2002). This is noteworthy, because activated platelets release ADP which potentiates the effect of other agents including weak agonists (e.g. 5HT or adrenaline) (Daniel *et al.,* 1998; Gachet, 2001b). It may be a disadvantage that a commonly used inhibitor like aspirin does not exert a more powerful effect on ADP-induced platelet responses.

It was also detected that when combination therapy (clopidogrel plus aspirin) was used, ADP-induced aggregation was inhibited to a greater extent than with clopidogrel alone. Therefore, combination therapy is more effective than monotherapy even when ADP-induced aggregation is assessed.

Either drug alone did not inhibit SPA. However, SPA was significantly inhibited with combination therapy (clopidogrel plus aspirin). SPA (in PRP) predicts a higher risk of coronary events and mortality in patients who survived a recent MI (Trip *et al.,* 1990; Wallace *et al.,* 1998).

There was a significant inhibition of ADP (0.4 μmol/l)-induced PSC with clopidogrel but not with aspirin. This lends further evidence to the concept that clopidogrel

exerts a more specific inhibitory action on ADP-induced platelet responses as compared to aspirin (Drouet *et al.,* 1998; Duerschmied *et al.,* 2010; Foster *et al.,* 2001; Gachet, 2001a). However, with the higher ADP concentration (0.8 μmol/l), some inhibition of PSC with aspirin only was observed. Nevertheless, clopidogrel remained the more potent inhibitor of PSC even at this high concentration of ADP. It is possible that aspirin-induced inhibition of PSC is only visible when the increase in MPV is substantial. ADP-induced PSC was more obviously inhibited with combination therapy than with monotherapy. Another study done in relation to this one, previously reported significant inhibition of ADP-induced PSC following a loading dose of clopidogrel in patients with PAD (Matsagas and **Jagroop** *et al.,* 2003). It was also demonstrated, that a $P2Y_{12}$ receptor inhibitor (AR-C66931MX) added *in vitro* inhibited the early phase of PSC induced by ADP (**Jagroop** *et al.,* 2003a).

Either drug alone or in combination did not significantly inhibit 5HT-induced aggregation in whole blood. However, another study showed that clopidogrel not only inhibited ADP-induced platelet aggregation, but also reduced platelet aggregation induced by a thromboxane mimetic (U46619) or 5HT in dogs (Yao *et al.,* 1992). The fact that in this study, a dose of 75 mg/day of oral clopidogrel was used, as compared to 10 mg/kg i.v. bolus plus 2.5 mg/kg/h infusion (for 3 h) may be a contributing factor to the inconsistency between these studies(Yao *et al.,* 1992). Apart from the greater drug availability via the i.v. route, the equivalent dose of clopidogrel (75 mg orally, once daily) in these patients (average weight: 75 kg) is much lower. However, when we used a loading dose of clopidogrel we did see inhibition of 5HT-induced aggregation (Matsagas and **Jagroop** *et al.,* 2003).

In contrast to 5HT-induced aggregation, 5HT-induced PSC was significantly inhibited by either drug alone (at 30 sec) or both drugs in combination. This may be because PSC is a more sensitive technique of assessing platelet activation. Much lower doses of agonists are required to induce PSC (*see Appendix A: PSC reference list authored by* ***IA Jagroop****;* Barradas *et al.,* 1992) than are necessary for platelet aggregation (*see Appendix B: platelet aggregation reference list authored by* ***IA Jagroop)***. For example, 0.5 μmol/l of 5HT was used to stimulate PSC as compared to 5-10 μmol/l, which was required to induce aggregation. It has also previously been reported that a significant inhibition of 5HT-induced PSC after a loading dose (300 mg) of clopidogrel in patients with PAD (Matsagas, **Jagroop** *et al.,* 2003).

Several trials evaluated the benefit and safety of clopidogrel alone or with aspirin for the prevention of cardiovascular events (Bakhru *et al.,* 2008; Mohler, III, 2009; Squizzato *et al.,* 2011). For example, CAPRIE (n = 19,185; Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events), where the PAD subgroup considered 6,452 patients and found that clopidogrel significantly (P = 0.0028) reduced (by 23.8%), the relative risk of vascular events compared with aspirin (1996, *no authors listed*). However, this subgroup analysis is limited by the fact that it was not included in the original CAPRIE protocol.

CURE (Clopidogrel in Unstable angina to prevent the Recurrent ischemic Events) (Yusuf *et al.,* 2001) was a large (n = 12,562) study to determine if acute and long-term treatment with the combination of clopidogrel and aspirin is superior to aspirin alone in patients with acute coronary syndrome. Combination therapy proved to be more effective than aspirin alone (Yusuf *et al.,* 2001).

The CREDO trial (Clopidogrel for Reduction of Events During Observation) (Steinhubl *et al.,* 2002) considered 2116 patients after percutaneous coronary intervention, and evaluated the effect of clopidogrel (75 mg/day and a loading dose of 300 mg) in addition to aspirin. CREDO showed that long-term (1 year) clopidogrel plus aspirin reduced the risk of ischaemic events, and that a longer interval (> 6h) between the loading dose of clopidogrel and PCI may also reduce events (Steinhubl *et al.,* 2002).

The efficacy of aspirin mono-therapy is well documented by a multitude of trials (2002, *no authors listed*). Furthermore, when patients were put on aspirin the platelet half-life was prolonged. In addition, a recent meta-analysis concluded that platelet inhibitors significantly reduce events in patients with PAD (2002 *no authors listed*).

From the present study we conclude that clopidogrel is a more powerful platelet inhibitor than aspirin in patients with PAD. This conclusion is compatible with the CAPRIE subgroup findings mentioned above (1996, *no authors listed*). Moreover, the clopidogrel and aspirin combination was more potent than either drug alone (Bakhru *et al.,* 2008; Mohler, III, 2009;Mohler, III *et al.,* 2003; Squizzato *et al.,* 2011). Whether this translates into additional clinical benefit in PAD is not established. Nonetheless, the results of CURE (Yusuf *et al.,* 2001) and CREDO (Steinhubl *et al.,* 2002) suggest that such additional inhibition is beneficial.

Any increased platelet inhibition needs to be balanced against a potential greater risk of bleeding. These issues can only be clarified by an extensive trial in patients with PAD. However, if combination therapy (clopidogrel plus aspirin) is proven to be more effective than mono-therapy (clopidogrel or aspirin) then the benefits may be considerable because

PAD is both common and associated with an increased risk of vascular events (Barradas *et al.,* 1994b; Matsagas *et al.,* 2002; Reininger *et al.,* 1999; Robless *et al.,* 2001). Until such evidence becomes available, using either drug alone or in combination in PAD, must remain the decision of the prescribing physician. The decision may be influenced by vascular events occurring while on mono-therapy or by intolerance to aspirin.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

Both ADP Receptors, $P2Y_1$ and $P2Y_{12}$, Play a Role in Controlling Shape Change in Human Platelets

4.1 (i) AIM, BACKGROUND AND HYPOTHESIS

Aim: The aim of this study was to assess the role of platelet purinergic receptors (i.e. $P2Y_{12}$, $P2Y_1$ and $P2X_1$) on PSC.

Background: Clopidogrel acts via $P2Y_{12}$ purinergic receptors. Furthermore, in my previous studies this drug, when administered in vivo, had an inhibitory effect on PSC. Thus, I wanted to investigate the involvement of $P2Y_{12}$ purinergic receptors as well as others, namely $P2Y_1$ and $P2X_1$ on the PSC. Others have implicated the $P2Y_1$ purinergic receptor in PSC, with the use of the less sensitive method of optical transmission aggregometry. Further evidence suggests that the measurements that were thought to represent PSC, actually showed the formation on platelet microaggregates. Thus, using my more sensitive and quantitative method to assess PSC (i.e. a channelyzer), it is of interest to show if the purinergic receptors $P2Y_1$, $P2Y_{12}$ and $P2X_1$ do play a role in PSC. This can be done with the use of specific purinergic receptor inhibitors (MRS2179 for $P2Y_1$, ARC 69932IMX for $P2Y_{12}$ and TNP-ATP for $P2X_1$).

Hypothesis: My hypothesis was that several platelet purinergic receptors can influence PSC. Moreover, my method to assess PSC would be able to determine the effectiveness (IC_{50}) of purinergic receptor inhibitors.

4.1 (ii) INTRODUCTION

Purinergic receptors (Ralevic et al, 1998) are potential targets for anti-thrombotic drugs, since ADP plays a crucial role in platelet activation (Burnstock, 1989). Two types of ADP receptors, $P2Y_1$ and $P2Y_{12}$, contribute to platelet aggregation (Li *et al.,* 2010; Remijn *et al.,* 2002; Storey *et al.,* 2001). The $P2Y_1$ receptor is involved in the initiation of aggregation by mobilizing calcium stores (Storey *et al.,* 2001). The $P2Y_{12}$ receptor is necessary for the completion and amplification of aggregation by inhibiting adenylyl cyclase activity (Gachet, 2008; Gachet, 2001b; Storey, 2001). The $P2X_1$ receptor is also present but by the year 2001 its role, in terms of platelet function, had not yet been defined (Gachet, 2001b). However, present knowledge suggests that the identified platelet $P2X_1$ receptor may be a target in the management of thrombosis, and its inhibition is potentially capable of platelet function modulation (Hu *et al.,* 2010).

The ATP contained in the platelet dense granules is released upon platelet activation into the extracellular space of the platelet (Hu *et al.,* 2010). Up until about 30 years ago, the role of ATP in haemostasis was vague. It is now understood, that the extracellular ATP can induce platelet activation signals, upon interaction with specific receptors that belongs to the P2X class of non-selective ATP-gated cation channels (North, 2002). MacKenzie *et al.* (MacKenzie *et al.,* 1996) demonstrated ATP-operated cation channel in platelets, which was linked to $P2X_1$ receptor and thus indicated that there was a role for ATP in platelet activation. Vial *et al.* (Vial *et al.,* 1997) suggested that there is a unique presence of $P2X_1$ receptors in human platelets and that this ion channel was the sole ATP-operated P2-receptor on platelets.

The initial functional response of platelets to ADP is shape change. These cells undergo cytoskeletal re-organisation resulting in spheration, contraction and generation of pseudopodia (Daniel *et al.,* 1998). PSC precedes aggregation and, *in vitro*, this process is essentially aspirin resistant (*see Appendix A: PSC reference list authored by* ***IA Jagroop****)*. Several studies that assessed PSC used light transmission aggregometery (Ehrman, 1980; Maurer-Spurej, 2001; Wilde, 2000). This technique is not as sensitive as using a high-resolution channelyzer (*see Appendix A: PSC reference list authored by* ***IA Jagroop****)*. Therefore, aggregometry may not have detected small variations in platelet size.

More deficiencies can be attributed to the optical method used to assess PSC. Indeed, Maurer-Spurej (2001) and others (Gabbasov, 1989; Maurer-Spurej, 2001) proposed that a decrease in light transmission actually represents the formation of platelet-microaggregates rather than PSC. We have shown, using a high-resolution channelyzer, that an increase in MPV as an indicator of PSC occurs concomitantly with morphological changes seen by electron microscopy (**Jagroop** *et al.,* 2000a).

Since the channelyzer method used in this study is reproducible and sensitive (*see Appendix A: PSC reference list authored by* ***IA Jagroop***), it was used to evaluate the role of the $P2Y_1$, $P2Y_{12}$ and $P2X_1$ receptors in mediating human PSC. In order to achieve this objective, the following specific purinergic receptor antagonists: MRS 2179 for $P2Y_1$ receptors (Boyer, 1998), AR-C69931MX for $P2Y_{12}$ receptors (Humphries, 1994), and TNP-ATP for $P2X_1$ receptors (Virginio, 1998) were used.

4.2 METHODS

Collection of blood and MPV measurement were carried out (as outlined in chapter 2, General Methods), and any alterations to these general methods are described below.

4.2.1 Obtaining an IC_{50} is for the $P2Y_1$ ADP Receptor Antagonist

PRP was prepared from 7 healthy volunteers (6 men and 1 woman; mean age: 38 years, range: 26 – 57) (as outlined in chapter 2, General Methods). PRP was stimulated with ADP, at a final concentration (fc) of 0.2 or 0.4 μmol/l for 30 sec and the platelet MPV was assessed using a Coulter ZM 34 counter, coupled to a C-256 channelyzer. This dose of ADP was selected to cause a 5 to 15% increase in MPV when compared with the physiological saline (control). This range was selected to provide a sub-maximal increase that is within the detection capacity of the high-resolution channelyzer, and can still allow for any inhibitory responses with antagonists to be detected. Saline control was added to samples to match those of the agonist (as 10 - 50 μl volumes) (*see Appendix A: PSC reference list authored by* ***IA Jagroop).*** In order to obtain an IC_{50} for the $P2Y_1$ ADP receptor antagonist**,** MRS 2179, PRP was incubated for 1 min with increasing doses of MRS 2179 ranging from 0.62 - 20 μmol/l in each of the 7 individuals, before adding ADP. These increasing doses of MRS 2179 were used, until the measured MPV value returned to that observed for the saline control (see figure 4.1).

4.2.2 The Effect of the $P2Y_1$ Receptor Antagonist on a Combination of ADP- and 5HT-induced PSC

PRP was prepared from 7 healthy volunteers (6 men and 1 woman; mean age of 38 years, range: 26 – 57) (as outlined in chapter 2, General Methods). PSC was stimulated with ADP only (fc 0.2 μmol/l), 5HT only (fc 0.03 - 0.25 μmol/l), and then ADP combined with 5HT. The increase in MPV induced by either ADP or 5HT was of the order of 1 – 3%. This range was selected to provide a sub-maximal increase that is within the detection capacity of the high-resolution channelyzer and can still allow for an additive of synergistic effect. For each individual, the dose of MRS 2179 that resulted in 100% inhibition of ADP-induced PSC (as described in section 4.2.1 above) was determined. This maximum dose of MRS 2179 ranged from fc 5 to 20 μmol/l. The selected dose was then incubated with PRP for 1 min before adding ADP combined with 5HT, the MPV was assessed 30 sec later.

4.2.3 The Effect of the $P2Y_{12}$ ADP Receptor Antagonist on PSC

PRP was prepared (as outlined in chapter 2, General Methods), from 12 healthy volunteers (7 men and 5 women; mean age of 32 years, range: 23 – 54). ADP alone (fc. 0.2 - 0.4 μmol/l) then ADP together with AR-C69931MX (10 μmol/l) was added to PRP. The MPV was analyzed (as described in Chap 2) 10, 15 and 30sec after adding these agents.

4.2.4 The Effect of a $P2X_1$ ADP Receptor Antagonist (TNP-ATP) on PSC

PRP was prepared from 7 healthy volunteers (6 men and 1 woman; mean age of 38 years, range: 26 – 57) (using the method described in chapter 2). PRP was stimulated with

ADP (fc) 0.2 or 0.4 μmol/l for 30 sec and the MPV assessed. This dose was selected to cause a 4 - 12% increase in MPV. This range was selected to provide a sub-maximal increase that is within the detection capacity of the high-resolution channelyzer and can still allow for any inhibitory responses with antagonists. To determine if there were any responses with TNP-ATP, PRP was incubated for various times (30 sec - 3 min) with a range of doses of TNP-ATP (26 - 102 μmol/l) before the addition of ADP (n = 7), or a combination of ADP plus 5HT (n = 5).

4.2.5 The Effect of a $P2X_1$ ADP Receptor Agonist α,β-methylene ATP (α,βmeATP) on PSC

PRP was prepared from 7 healthy volunteers (3 men and 4 women; mean age of 36 years, range: 28 – 56) (as outlined in chapter 2, General Methods). PRP was stimulated with α,βmeATP in increasing doses ranging from 0.5 μmol/l to 20 μmol/l. PRP was incubated with α,βmeATP for various times (10, 15 and 30 sec) and the MPV was assessed. These times were selected on the basis of previous research showing that the response of this receptor is very rapid (Takano *et al.,* 1999; Vial *et al.,* 2002).

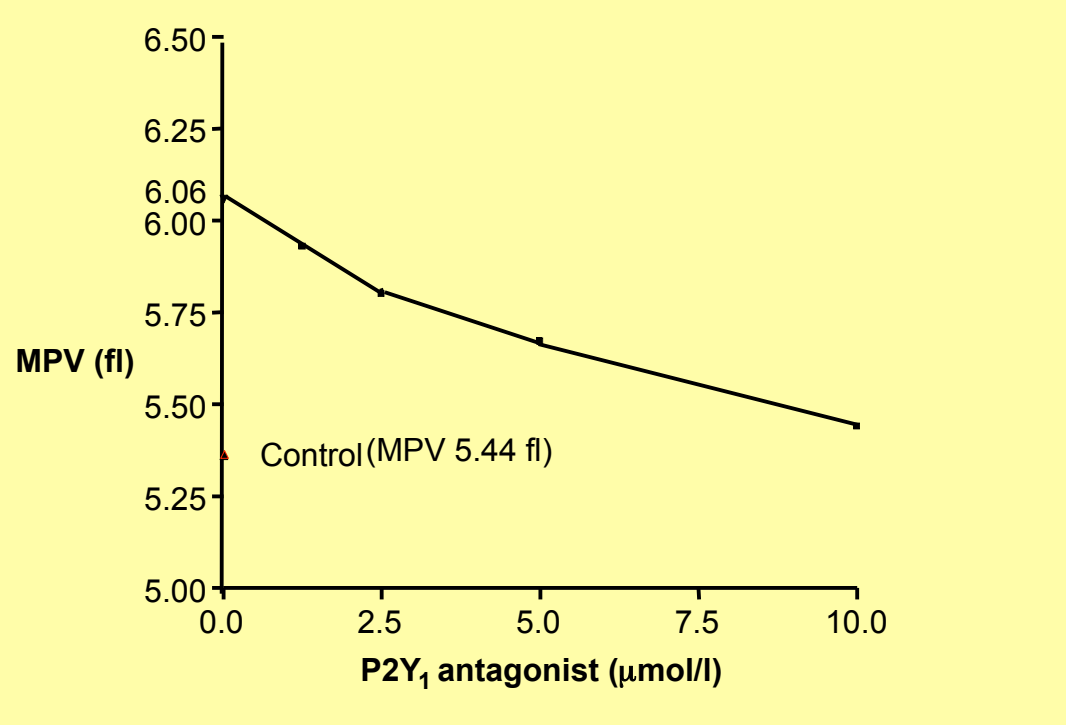
4.2.6. Statistical Analysis: The results are presented as median and range. Statistical comparisons are by Wilcoxon paired test.

4.3 RESULTS

4.3.1 Obtaining an IC_{50} for the $P2Y_1$ ADP Receptor Antagonist

MRS 2179 inhibited ADP-induced PSC by 100% in each of the 7 subjects tested. The median IC_{50} for MRS 2179 was 3.16 (1.06 - 10.25 μmol/l). Therefore, the range for IC_{50} of MRS 2179 showed a 10-fold variation when all 7 subjects were considered. A representative dose response curve is shown in figure 4.1 (next page). In this subject, the control MPV is 5.44 fl. By inducing PRP with ADP, the MPV increases to 6.06 fl which is inhibited by increasing concentrations of MRS 2179.

Figure 4.1: A representative curve showing the IC_{50} of MRS 2179 (3.33 μmol/l) on ADP-induced PSC



A representative dose response curve of one subject showing that an IC_{50} (3.33 μmol/l)) of a $P2Y_1$ receptor antagonist (MRS 2179) was obtained. In this subject, the control MPV was 5.44 fl. By inducing PRP with ADP, the MPV increased to 6.06 fl, which was inhibited by increasing concentrations of MRS 2179.

4.3.2 The effect of the $P2Y_1$ ADP Receptor Antagonist on a Combination of ADP and 5HT- induced PSC

The ADP-induced increase in MPV was of the order of 2% greater than that for the saline control. For 5HT, the increase was of the order of 1%. In combination, ADP plus 5HT caused a synergistic increase in MPV of 8%. This increase was significantly (P = 0.01) inhibited by MRS 2179 *(see table 4.1),* (at a dose that abolished PSC-induced by ADP alone).

Table 4.1 Effect of MRS 2179 on a Combination of 5HT plus ADP-induced PSC

	MPV (fl), median (and range)
Saline	6.18 (5.36 - 6.44)
ADP	6.31 (5.6 - 6.88)
5HT	6.25 (5.55 - 6.82)
ADP + 5HT	6.69 (6.18 - 7.14)
MRS 2179 with ADP plus 5HT	6.44 (5.93 - 6.69)*

* ADP plus 5HT vs MRS 2179 with ADP plus 5HT, (n = 7, in each group), **P = 0.01**
The results are presented as median and range. Statistical comparisons are by Wilcoxon paired test.

4.3.3 The effect of the $P2Y_{12}$ ADP Receptor Antagonist on PSC

ADP-induced PSC showed a 4 - 6% increase in MPV (fl) when compared with the saline controls. The ADP (0.2 μmol/l)-induced increase in MPV was significantly inhibited by AR-C69931MX (10 μmol/l) *(see table 4.2).* AR-C69931MX also inhibited ADP-induced PSC up to 5 min, but with increasing time, the inhibition was less (figure 4.2.) This same trend in inhibition was seen for 0.4 μmol/l.

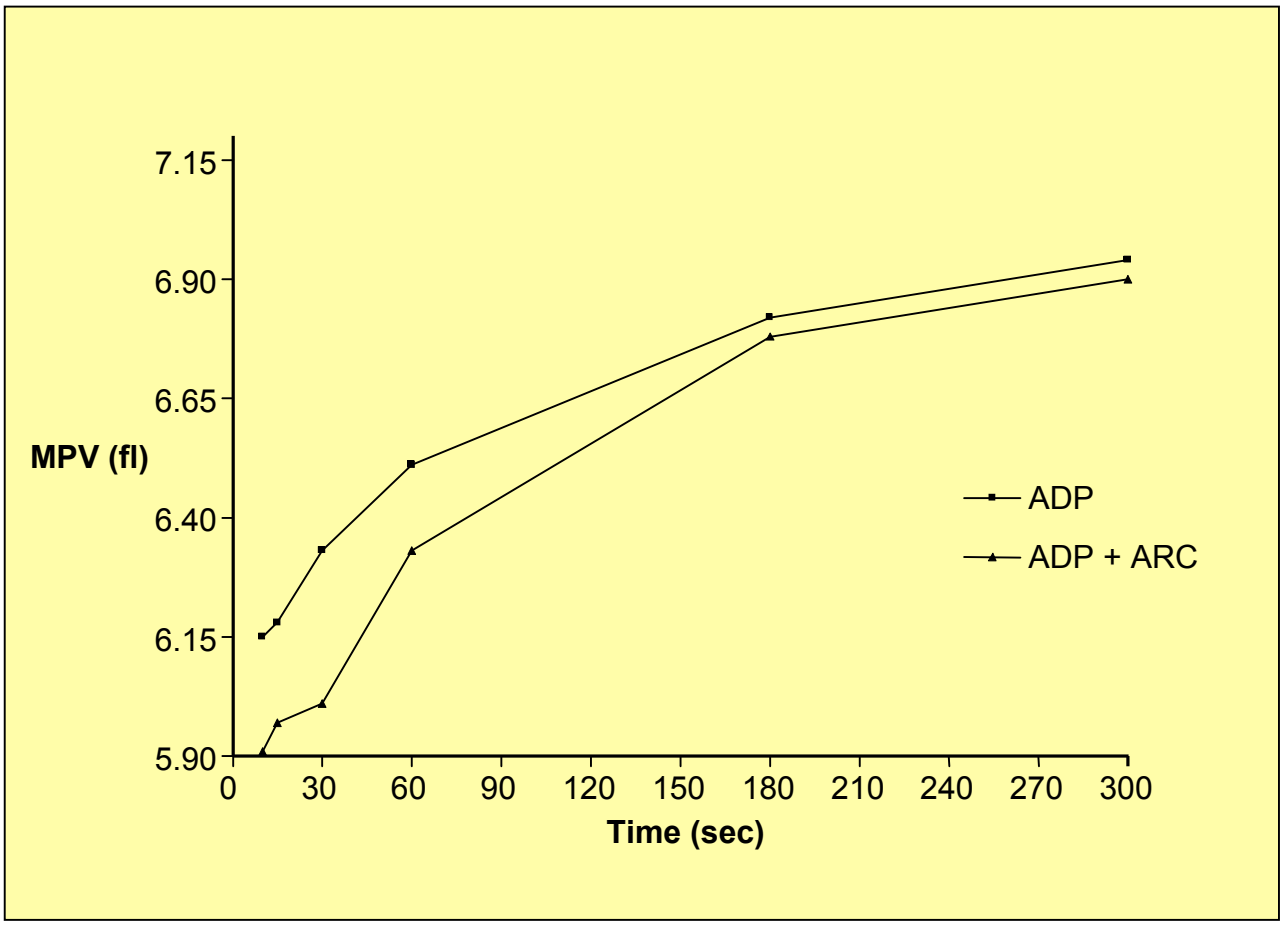
Although AR-C69931MX inhibited ADP-induced PSC 30 sec after its initiation, the inhibitory effect gradually decreased so that it was no longer evident at 5 min.

Table 4.2 The Effect of AR-C69931MX on ADP-induced PSC

Time (sec)	10 sec	15 sec	30 sec
Saline	5.88 (4.78 - 6.95)	5.85 (4.95 - 6.76)	5.90 (4.78 - 6.69)
ADP	6.12 (5.48 - 7.01)	6.19 (5.48 - 7.07)	6.24 (5.55 - 7.20)
AR-C69931MX + ADP	5.94 (5.42 - 6.76)	6.08 (5.47 - 6.84)	6.10 (5.48 - 7.01)
P value	**P = 0.009**	**P = 0.001**	**P = 0.010**

The P values are a comparison of ADP vs ADP plus AR-C69931MX at 10, 15 and 30 sec. Results are expressed as MPV (fl), n = 12. The results are presented as median and range. Statistical comparisons are by Wilcoxon paired test.

Figure 4.2 A representative curve (n = 2) of the effect of AR-C69931MX (10 μmol/l) on ADP (0.2 μmol/l)-induced PSC



4.3.4 The effect of a $P2X_1$ Receptor Antagonist (TNP-ATP) on PSC

There was no significant effect (n = 7) on ADP-induced shape change, (6.31; 6.12 - 6.88 fl) after adding TNP-ATP (6.36; 6.18 - 7.01 fl).

There was also no significant effect (n = 5) of TNP-ATP (6.61; 6.12 - 6.95 fl) on PSC-induced by the combination of ADP plus 5HT (6.57; 6.18 - 7.07 fl).

4.3.5 The effect of a $P2X_1$ ADP Receptor Agonist (α,βmeATP) on PSC

There was no effect of α,β-methylene ATP (α,βmeATP) on MPV at any of the time points, or concentrations assessed (e.g. saline control at 10 sec, 5.87; 5.36 - 6.66 fl vs α,βmeATP at 10 sec, 5.87; 5.36 - 6.63 fl).

4.4 DISCUSSION

In this study, using a $P2Y_1$ receptor-blocker, 100% inhibition of ADP-induced PSC was achieved. Others have also shown that ADP-induced PSC, occurs via the $P2Y_1$ receptor (Chang *et al.,* 2010; Mangin *et al.,* 2004; Storey *et al.,* 2001). However, some of these authors (Storey *et al.,* 2001) measured PSC using light transmission aggregometry and it has been suggested that the aggregometer tracing taken to represent PSC rather reflects the formation of platelet micro-aggregates (Maurer-Spurej, 2001). The $P2Y_1$ receptor is also essential for platelet aggregation (Baurand, 2001; Raboisson, 2002). Therefore, antagonists to this receptor may become useful anti-platelet agents (Raboisson, 2002).

In these experiments, the IC_{50} for MRS 2179 varied between 1.06 - 10.25 μmol/l (i.e. a 10-fold variation in IC_{50} when all 7 subjects were considered); the median IC_{50} was 3.16

μmol/l. To my knowledge, an IC_{50} has not been derived before for a $P2Y_1$ receptor-blocker using the human PSC phenomenon. An IC_{50} for $P2Y_1$ receptor-blockers can now be derived using a channelyzer because of the high-resolution (0.07 fl) and reproducibility of this technique (**Jagroop** *et al.,* 1996; **Jagroop** *et al.,* 2000c; **Jagroop** *et al.,* 2000a; *see Appendix A: PSC reference list authored by* ***IA Jagroop*** *et al.).*

In some of these experiments, the effect of ADP on a preparation of PRP was observed. It is know that the initial functional response to ADP is shape change, where platelets go through cytoskeletal reorganization, ending up in spheration, contraction and pseudopodia generation (Fox, 2001). It is thought that phosphorylation of the myosin light chain (MLC) may have a central role in PSC (Daniel *et al.,* 1998). Phosphorylation may happen when there is an elevation of calcium through calmodulin-dependent activation of MLC-kinase (MLCK) (Wilde *et al.,* 2000). Moreover, others have shown that in another cell type, Rho-kinase directly phosphorylates MLC and inactivates MLC-phosphatase (Essler, 1998; Kureishi, 1997). This resulted in an increase in MLC phosphorylation. When considering platelets, it has been observed that the Rho-kinase, RhoA (a Rho-kinase) and the myosin binding subunit of MLC-phosphatase tends to create a complex, which ends up as the phoshorylation of MLC-phosphatase and a reduction in its activity (Suzuki, 1999).

In this work, it was demonstrated that AR-C69931MX inhibited ADP-induced PSC, in a time-dependent manner, in samples collected from healthy volunteers. This is the first study that actually quantifies PSC inhibition by this or any other $P2Y_{12}$ receptor blocker. Others have shown, using an optical aggregometer and washed platelets, that another $P2Y_{12}$ receptor antagonist (AR-C66096) caused a more rapid reversion of platelets to the discoid

form (Wilde, 2000). There was also evidence that PSC was inhibited by cAMP. This finding may explain why blocking the $P2Y_{12}$ receptor results in inhibition of PSC, since stimulating the $P2Y_{12}$ receptor inhibits cAMP synthesis (Wilde, 2000).

When using two agonists (i.e. ADP plus 5HT) in combination in the presence of the maximal dose of the $P2Y_1$ receptor-blocker, the mean MPV was greater than that with 5HT alone. This finding suggests that the unblocked $P2Y_{12}$ purinergic receptor can enhance the 5HT-mediated PSC response. Therefore, it would seem that the $P2Y_{12}$ receptor is involved when ADP acts as a co-stimulus in the presence of low concentrations of other agonists, as previously suggested (Gachet, 2001).

The first observations of the functional involvement of the $P2X_1$ receptor in platelets were seen in 1995 (Humphrey *et al.,* 1995; Surprenant *et al.,* 1995). Moreover, the $P2X_1$ receptor could be implicated in PSC (Rolf, 2001). However, in spite of the already demonstrated functionality, many investigators failed to find any physiological activity for that purinergic receptor other than calcium influx (Daniel, 1998; Vial, 1997). It was indicated that the physiological role of $P2X_1$ receptor in platelet function, might have remained concealed by other investigators, due to the rapid $P2X_1$ receptor desensitization when the platelets are prepared *in vivo* (Hu *et al.,* 2010; Rolf *et al.,* 2001).

An effect of the $P2X_1$ receptor on PSC (after using both a specific antagonist and agonist) was absent. This supports the others who show that the receptor is involved with very rapid actions (Vial, 1997). In addition, the sampling times used in these experiments, was limited by the capacity of human operators. Citrated PRP was used, and this may have masked any Ca^{2+}-mediated effect.

This work supports the theory that the $P2X_1$ receptor becomes rapidly desensitized and it is therefore difficult to assess its effect on platelets *in vitro* (Gachet, 2001). Rolf *et al.* were able to prevent this desensitization of $P2X_1$ receptor by using a high concentration of apyrase (ATP-diphosphohydrolase E.C.3.6.1.5.) (Rolf *et al.,* 2001). The selective $P2X_1$ receptor agonist α,β-meATP, induced a rapid Ca^{2+}-influx that was associated with a transient shape change in human platelets. There is evidence suggesting that the morphological changes mediated by $P2X_1$ receptor activation, involves rapid pseudopodia formation, a fast and reversible centralisation of secretory granules, platelet spearing and short filopodia formation (Toth-Zsamboki *et al.,* 2003; Vial *et al.,* 2003).

It was important to exclude an effect of the $P2X_1$ receptor on PSC within this experimental design because the $P2Y_1$ receptor-blocker used in these experiments may also exert some action on the $P2X_1$ receptor (Horner, 2005d; Gachet, 2005). However, any such interference is unlikely to have occurred because the $P2X_1$ receptor showed no effect on PSC (after using both a specific antagonist and agonist). Furthermore, in another experiment (rat vas deferens), MRS 2179 at concentrations of 100 μmol/l did not exert any action on the $P2X_1$ receptor (Horner, 2002). The maximal concentration of MRS 2179 we used was 20 μmol/l.

This channelyzer method was previously used to demonstrate the action of drugs (added *in vitro* at therapeutic concentrations) on platelets (**Jagroop** *et al.,* 1996; **Jagroop** *et al.,* 2000c; **Jagroop** *et al.,* 2000a; *see Appendix A: PSC reference list authored by* ***IA Jagroop*** *et al.)* when other methods only detected an effect at unrealistically high concentrations. These findings are of clinical relevance because PSC is an early phase of

platelet activation that precedes aggregation and it is essentially aspirin resistant (**Jagroop** *et al.,* 1996; **Jagroop** *et al.,* 2000c; **Jagroop** *et al.,* 2000a; *see Appendix A: PSC reference list with* ***IA Jagroop*** *et al.)* at least *in vitro*.

These findings suggest that $P2Y_{12}$ receptor antagonists are clinically useful since they inhibit both early and late platelet activation. This concept is supported by clinical trials (Puri, 1997). For example, there is extensive trial-based evidence showing that clopidogrel (a $P2Y_{12}$ receptor antagonist) significantly decreases the risk of vascular events when administered alone or in combination with aspirin (1996 *no authors listed*; Yusuf, 2001). Unfortunately, the inhibitory effect of clopidogrel cannot be assessed *in vitro* because it requires to be metabolized before exerting its actions (Mikhailidis and **Jagroop**, 1998; **Jagroop** *et al.* 2004). Whether $P2Y_1$ or $P2Y_{12}$ receptor antagonists will prove superior to other anti-platelet agents in certain disease states, remains to be proven (Robless, 2001). In addition, there is a need to establish whether the contribution of different platelet purinergic receptors is altered in various types of vascular disease.

Assessing the effect of clopidogrel on the $P2Y_{12}$ receptor is limited because this anti-platelet agent is inactive *in vitro* (Savi, 2000). Clopidogrel needs to be metabolized by the liver before becoming active. At least one of the active metabolites of clopidogrel has been purified and shown to be active *in vitro* (Savi, 2000). However, the production of this metabolite requires incubation with human liver microsomes followed by a purification process. In addition, this metabolite is highly unstable (Savi, 2000). It follows that the use of this metabolite is limited.

In conclusion, platelet receptor antagonists will exert different actions on human platelets. Both the $P2Y_1$ and $P2Y_{12}$ receptors appear to be involved in mediating PSC in human platelets (**Jagroop** *et al.*,, 2002d; **Jagroop** *et al.,* , 2003). The channelyzer method provides a useful tool to assess the platelet inhibitory action of these drugs (**Jagroop** *et al.,* 1996;**Jagroop** *et al.,* 2000c; **Jagroop** *et al.,* 2000a; *see Appendix A: PSC reference list authored by* ***IA Jagroop*** *et al.)*.

4.5 ACKNOWLEDGEMENTS

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CHAPTER 5

The Effect of Tirofiban on Fibrinogen- and Agonist-induced Aggregation and Platelet Shape Change

5.1 (i) AIM, BACKGROUND AND HYPOTHESIS

Aim: The aim of this study was to assess if a GPIIb/IIIa receptor antagonist, tirofiban, had an effect on fibrinogen- and agonist-induced PSC, an early phase of platelet activation that is essentially aspirin resistant.

Background: It is usual for PAD patients, to be on antiplatelet therapy like aspirin or clopidogrel (or even both). However, these patients may be resistant to aspirin and/or clopidogrel. It therefore makes sense to consider other antiplatelet drugs that act via other receptors. Hence, in this study I investigated the effect of a GPIIb/IIIa receptor antagonist, tirofiban, on fibrinogen- and agonist-induced PSC and aggregation. Raised plasma fibrinogen levels and platelet hyperactivity have been shown to be linked with vascular events. One way to inhibit platelets is to block the platelet GPIIb/IIIa receptor, which binds circulating fibrinogen or von Willebrand factor and cross links platelets at the final common pathway to platelet aggregation. It is not known if fibrinogen affects PSC in human (or animal) platelets. Therefore, I also assessed this process. The use of the GPIIb/IIIa antagonists (like tirofiban, abciximab and eptifibatide) has not been as widespread as previously forecasted, they appear to have low bioavailability, and have the ability to exert partial agonism. This study may lend some evidence as to why that is.

Hypothesis: My hypothesis was that GPIIb/IIIa antagonists like tirofiban do not affect PSC and that this may be a reason for their diminished efficacy.

5.1 (ii) INTRODUCTION

There is evidence linking platelet hyperactivity with vascular events (Valgimigli, 2010; Robless *et al.,* 2003). For example, patients with more activated platelets had a higher incidence of myocardial infarction (MI) and early restenosis (1997 *no authors listed*). Platelet activation may also play a role in the pathogenesis of complications after percutaneous coronary intervention and stent implantation (Valgimigli *et al.,* 2010; Yip *et al.,* 2005). Patients with hyperactive platelets may benefit more from intensive anti-platelet therapy as platelet inhibitors significantly reduce the risk of vascular events (Ferguson, 2006; Robless *et al.,* 2003).

One way to inhibit platelets is to block the platelet membrane glycoprotein (GP) IIb/IIIa receptor, which binds circulating fibrinogen (fib) or von Willebrand factor (vWF) and crosslinks platelets as the final common pathway to platelet aggregation (Huang *et al.,* 2004). Activated platelets express the integrin αIIb β3 (also known as the activated platelet membrane glycoprotein receptor GPIIb/IIIa) on their surface, which binds fib (Kakafika *et al.,* 2007). Raised plasma fib concentrations increases the risk of vascular events (Mikhailidis *et al.,* 1986). Moreover, platelet hyperactivity has also been reported in most of the conditions where plasma fib concentrations are increased (Kakafika *et al.,* 2007; Mikhailidis *et al.,* 1986; Tsiara *et al.* 2003).

GPIIb/IIIa receptor antagonists are potent inhibitors due to the critical role that fib binding plays in platelet aggregation (Huang *et al.,* 2004). To date, the US Food and Drug administration have approved 3 GPIIb/IIIa antagonists: abciximab (a chimeric monoclonal anti-body 7E3 Fab fragment), eptifibatide (a cyclic heptapeptide based on the KGD amino

acid sequence) and tirofiban (a non peptide tyrosine derivative) (Huang *et al.,* 2004; Jennings *et al.,* 2002).

Tirofiban hydrochloride, L-tyrosine-*N*-(butylsulfonyl)-*O*-[-4-(4-piperidinylbutyl)] monohydrochloride, is a potent and specific fib receptor antagonist, and is approved (under the name Aggrastat®) for use in the prevention of the progression of unstable angina to MI (Bukow *et al.,* 2006; Huang *et al.,* 2004; Jennings *et al.,* 2002) .

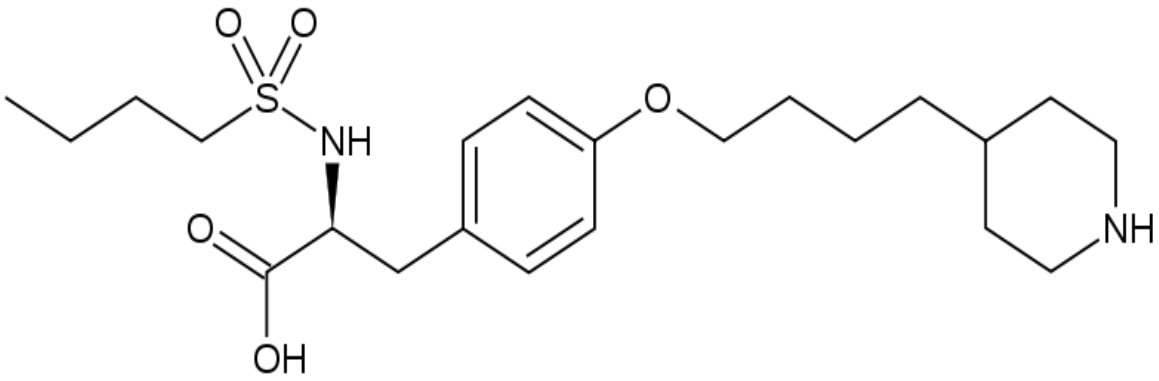

Figure.5.1 Molecular structure of tirofiban

Other intra-venous agents directed against the GPIIb/IIIa receptors include abciximab, eptifibatide and a non-peptide mimetic, lamifiban (Westerhout *et al.,* 2003). Clinical trials including more than 18,000 patients have shown the benefits of intra-venous GPIIb/IIIa blockade (Mak *et al.,* 1999a). Overall, at 30 days, 13 fewer deaths or MIs occurred for every 1000 patients treated in these trials. This favorable outcome was extended to 6 months, resulting in 16 fewer such events per 1000 patients treated. Importantly, these benefits were not accompanied by an excessive occurrence in bleeding complications or thrombocytopenia. The safety and efficacy of these GPIIb/IIIa antagonists

have been evaluated initially in patients undergoing percutaneous coronary intervention. Over 20,000 patients were enrolled in 9 major studies of abciximab, eptifibatide and tirofiban. The result of all these trials was the widespread use of GPIIb/IIIa antagonists (1994; 1997b; 1997a *no authors listed*).

In the present study, the effect of tirofiban on spontaneous platelet aggregation (SPA), as well as fib-, 5HT- and ADP-induced aggregation, was assessed. Various agonists were used alone and in combination. The rationale was that since ADP is an important aggregating agent, and raised plasma fib concentrations increases the risk of vascular disease, it would be useful to evaluate the inhibitory effect of tirofiban in this model. Also circulating levels of agonists like 5HT or ADP on its own may not have any effect on platelets but in combination may produce a synergistic harmful effect (e.g. stimulating vascular smooth muscle proliferation and inducing vasoconstriction in atheromatous coronary vessels) (Akopov *et al.,* 1992). The effect of tirofiban on a combination of fib and ADP at sub-threshold concentrations, was also investigated. Others have shown that optimal platelet aggregation occurs between pairs of agonists (e.g. ADP-epinephrine and epinephrine-collagen) even when each of the agonists is added to the other at sub threshold doses (Razi *et al.,* 2004).

Another study demonstrated that the platelet surface expression of the functional GPIIb/IIIa is required for 5HT release from delta-granules (Gobbi *et al.,* 2006). Therefore, the effect of tirofiban on 5HT-induced platelet aggregation was investigated.

The effect tirofiban on 5HT- and ADP-induced PSC an early phase of platelet activation, that precedes aggregation was also measured (**Jagroop** *et al.,* 1996; **Jagroop** *et*

al., 2000c; **Jagroop** *et al.,* 2000a; *see Appendix A: PSC reference list authored by* ***IA Jagroop*** *et al.,*). This work also investigated whether fib alone or in combination with another agonist affects PSC. To my knowledge, this has not yet been investigated using a high-resolution channelyzer.

5.2 MATERIALS - See Appendix D

5.3 METHODS

5.3.1 Whole Blood Aggregation

Venous blood was collected and whole blood (WB) aggregation was performed (as described in Methods Section, Chapter 2) as in previous studies **(Jagroop** *et al.,* 1996; **Jagroop** *et al.,* 2002b; **Jagroop** *et al.,* 2007; Robless *et al.,* 2003). Details of the volunteers used in each part of this study are given in the relevant results sections below. The effect of tirofiban on SPA was assessed after WB was stirred at specific time points (0, 5, 10, 15 and 20 min) with the following combinations, saline control/SPA; tirofiban only (50 ng/ml). The effect of tirofiban on fib only was also assessed when WB was stirred with fib only, and then tirofiban (500 ng/ml) plus fib. Then aggregation was measured at the same time points (0, 5, 10, 15 and 20 min) as above.

ADP (5 μM)-induced aggregation was also evaluated. WB was then incubated with tirofiban (500 ng/ml) for 5 min after which ADP (5 μM) was added and aggregation determined at the (0, 5, 10, 15 and 20 min) (see Chapter 2). Briefly, the blood samples were placed in siliconised aggregometer cuvettes and stirred using a Teflon-coated magnetic stirrer during the experiments. The samples were maintained at 37^0C in a Chronolog whole

blood aggregometer 540 (Coulter Electronics, Luton, Beds, UK) throughout the experiments. Blood samples from the aggregometer cuvettes were then presented to the Coulter® ACT Diff Analyzer (Coulter Electronics, Luton, Beds, UK) which allows open tube analysis and the platelet count determined. The results are expressed as % free platelet count. For example, if the baseline count was 300 $x10^9$/l and this fell to 200 $x10^9$/l at a later time then the % free count is 200/300 x 100 = 66.6%.

The effect of tirofiban on WB aggregation-induced by a combination of agonists at sub-threshold concentrations was measured over a specified time period (1, 3, 5, 10, 15 and 20 min). Thus, saline (control); ADP (1 μM); fib (0.5 g/l); a combination of ADP (1 μM) plus fib (0.5 g/l) was assessed. Since lower doses of agonists were used, the effect of the lower dose of tirofiban (250 ng/ml) on this combination by firstly incubating WB with tirofiban (250 ng/ml) for 5 min before adding the combination of ADP (1 μM) plus fib (0.5 g/l) was assessed.

To observe the effect of tirofiban on 5HT-induced aggregation, 5HT (5 μM) was added and aggregation assessed at the several time points (1, 3, 5, 10, 15 and 20 min) as previously described (Barradas et al.,, 1994). Then WB was incubated with tirofiban (500 ng/ml) for 5 min and 5HT (5 μM) was added before measuring aggregation again.

5.3.2 Median Platelet Volume Measurement

Venous blood collection, preparation of platelet rich plasma (PRP) and details of median platelet volume (MPV) measurement in are described in Chapter 2. The number of

volunteers used in each experiment is given in the relevant sections below. PRP was collected from 7 healthy volunteers, and was incubated with tirofiban (50 - 500 ng/ml) for 3-5 min without stirring and then either ADP (0.2 –0.4 μM) or 5HT (0.25- 0.5 μM)-induced PSC was measured after 30s and 1 min as outlined below. ADP- or 5HT- induced PSC was again assessed at the same concentrations but without tirofiban.

The effect of fib (1 – 8 g/l)-induced PSC with and without tirofiban (125 – 500 ng/ml) at various time points (between 30 s and 20 min) was also assessed. In addition, lower concentrations of fib (0.5 – 1.5 g/l) was used in combination with 5HT (0.25 μM) or ADP (0.2 μM). The MPV was assessed (after 30 sec and 1 min), using a Coulter ZM counter (electrical impedance method with a 70 μm diameter sampling tube orifice) coupled to a C-256 channelyzer (Coulter Electronics, Luton, Beds, UK) (as described in Chapter 2, section 2.4). The specific times for adding agonists and antagonists and were based on preliminary experiments and our previous publications (**Jagroop** *et al.,* 1996; **Jagroop** *et al.,* 2000c; **Jagroop** *et al.,* 2000a; *see Appendix A: PSC reference list authored by* ***IA Jagroop*** *et al.,*). Moreover, the concentration of agonists and sampling times to obtain a middle range response that could be either inhibited or enhanced were also determined in previous studies (**Jagroop** *et al.,* 1996; **Jagroop** *et al.,* 2000c; **Jagroop** *et al.,* 2000a; *see Appendix A: PSC reference list authored by* ***IA Jagroop*** *et al.,*).

The increase in MPV was taken as representing the PSC. Saline (control) was added to samples to match those of the agonist (as 10 - 50 μl volumes) at the same time points. The platelet count was monitored throughout the experiment to exclude PRP samples where significant (>5%) aggregation occurred.

5.3.3 Statistical Analysis and Presentation of Results

Statistical analysis was performed using GraphPad Prism Version 4 (Graph Pad Software, Inc, San Diego, California, USA). Comparisons were carried out using paired t-tests (or non-parametric tests) Results are expressed as mean and ± SD and a $P<0.05$ was considered significant. The results in the figures are expressed as median percentage free platelet count.

5.4 RESULTS

5.4.1 Whole Blood Aggregation

Tirofiban (50 ng/ml) significantly inhibited SPA at all time points (5 to 20 min). Table 5.1 shows statistical comparisons and figure.5.1 shows a representative curve of this effect.

The (2 g/l) concentration of fib demonstrated a similar trend of platelet aggregation over time as the saline control. In contrast, fib 4 g/l significantly increased platelet aggregation after 5 min ($P = 0.002$), 10 min ($P = 0.033$) and 15 min ($P=0.05$, n=8) (*see table 5.2*). Tirofiban (500 ng/ml) significantly inhibited the fib (4 g/l)-induced platelet aggregation. A similar pattern of aggregation and inhibition of aggregation was observed with fib 8 g/l and tirofiban (500 ng/ml) respectively. The addition of tirofiban to WB produced aggregation that was even less than that observed with the saline control (*table 5.2* shows statistical comparisons and figure.5.2 is a representative curve of this effect). There

was significant inhibition of ADP (5 μM)-induced platelet aggregation by tirofiban (500 ng/ml) at all time points *(see table 5.3*).

A lower concentration of fib (0.5 g/l) demonstrated a similar platelet aggregation pattern as the saline control. ADP (1μM)-induced platelet aggregation showed an expected transient significant increase in aggregation as compared with the saline control at 1 min and 3 min (96 ± 5 vs 39 ± 21, P = 0.001 and 92 ± 4 vs 71 ± 17, P = 0.035, respectively).

Beyond these sampling times there was aggregation but it was not significant in comparison with saline (see figure 5.3). The effect of a very low dose of ADP (1 μM) in combination with a very low dose of fib (0.5 g/l) on platelet aggregation followed a similar pattern as ADP over time with no significant additive aggregating effect (figure 5.3). Tirofiban (250 ng/ml) significantly inhibited platelet aggregation induced by this ADP plus Fib combination at all time points to a level that was even better than saline on its own (see table 5.4 for statistical analysis). There was an expected increase in 5HT (5 μM)-induced aggregation over time in comparison with the saline control. This aggregation was significantly inhibited by tirofiban (500 ng/ml) (*table 5* shows statistical comparisons).

Table 5.1 The Effect of a Lower Dose of Tirofiban (50 ng/ml) on SPA.

Time (min)	Saline alone (control)	Saline + tirofiban	**P value**
5	87 ± 8	99 ± 2	**0.006**
10	80 ± 14	99 ± 2	**0.01**
15	71 ± 13	98 ± 2	**0.001**
20	64 ± 19	98 ± 2	**0.005**

Results are expressed as mean and ± standard deviation and compare the percentage free platelet counts at different times. 7 healthy volunteers were evaluated. Spontaneous Platelet Aggregation (SPA, Saline Control).

Table 5.2 The Effect of Fibrinogen on SPA, and of Tirofiban on Fibrinogen-induced Platelet Aggregation over Time

Time (min)	Saline vs Fib	Fib vs Fib + Tirof
5	80 ± 10 vs 66 ± 6	66 ± 6 vs 86 ± 13
	P = 0.002	**P = 0.011**
10	72 ± 15 vs 57 ± 5	57 ± 5 vs 87 ± 11
	P = 0.03	**P = 0.0006**
15	68 ± 14 vs 48 ± 10	48 ± 10 vs 88 ± 10
	P = 0.04	**P = 0.0001**
20	56 ± 19 vs 45 ± 10	45 ± 10 vs 88 ± 12
	NS (0.23)	**P < 0.0001**

Results are expressed as mean and ± standard deviation and compare the percentage free platelet count. 8 healthy volunteers were evaluated.

Note: fib = fibrinogen (4 g/l); tirof = tirofiban (500 ng/ml); NS = not significant.
SPA = Spontaneous Platelet Aggregation (i.e. saline control)

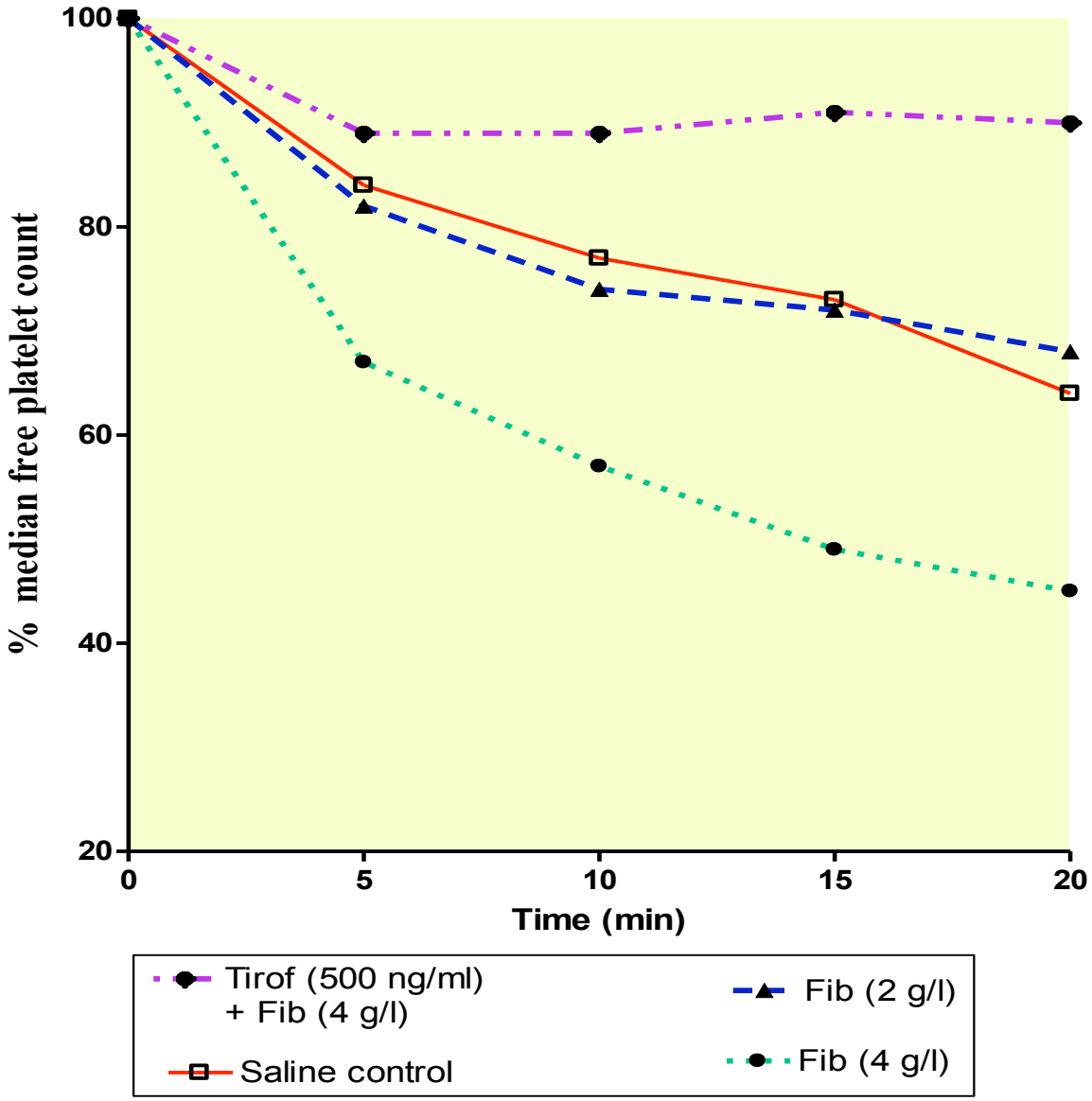

Figure 5.2: The effect of fibrinogen on platelet aggregation over time.

The representative curve shows the effect of fibrinogen (2 and 4 g/l) on platelet aggregation over time as compared with saline control. The inhibition of fibrinogen-induced aggregation by tirofiban (results are expressed as percentage median free platelet count, n=8).

Table 5.3 The Effect of Tirofiban on ADP-induced Platelet Aggregation over Time

Time (min)	Saline vs ADP	ADP vs ADP + Tirof
5	83 ± 11 vs 31± 13	31 ± 13 vs 86 ± 8
	P = 0.0001	**P< 0.0001**
10	72 ± 15 vs 30 ± 12	30 ± 12 vs 85 ± 10
	P = 0.0001	**P< 0.0001**
15	64 ± 14 vs 31 ± 13	31 ± 13 vs 87 ± 11
	P = 0.0001	**P< 0.0001**
20	54 ± 16 vs 34 ± 14	34 ± 14 vs 94 ± 7
	P < 0.007	**P< 0.0001**

Results are expressed as mean and ± standard deviation and compare the percentage free platelet count. 8 healthy volunteers were evaluated

Notes: ADP = adenosine diphosphate (5 μM); tirof = tirofiban (500 ng/ml).

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Table 5.4 The Effect of Tirofiban on the Combination of ADP plus Fibrinogen.

Time (min)	ADP + Fib **vs** ADP + Fib + Tirof	**P = Value**
1	38 ± 13 vs 90 ± 4	**<0.0001**
3	68 ± 16 vs 92 ± 5	**0.011**
5	70 ± 12 vs 92 ± 2	**0.003**
10	68 ± 11 vs 90 ± 5	**0.005**
15	66 ± 12 vs 93 ± 5	**0.002**
20	65 ± 11 vs 94 ± 6	**0.002**

Results show platelet aggregation for ADP (1 μM) plus fib (0.5 g/l) combination, compared with the effect of tirofiban (250 ng/ml) on this combination of agonists over time.
Results are expressed as mean and ± standard deviation and compare the percentage free platelet count. 7 healthy volunteers were evaluated.

Notes: ADP = adenosine diphosphate; fib = fibrinogen; tirof = tirofiban.

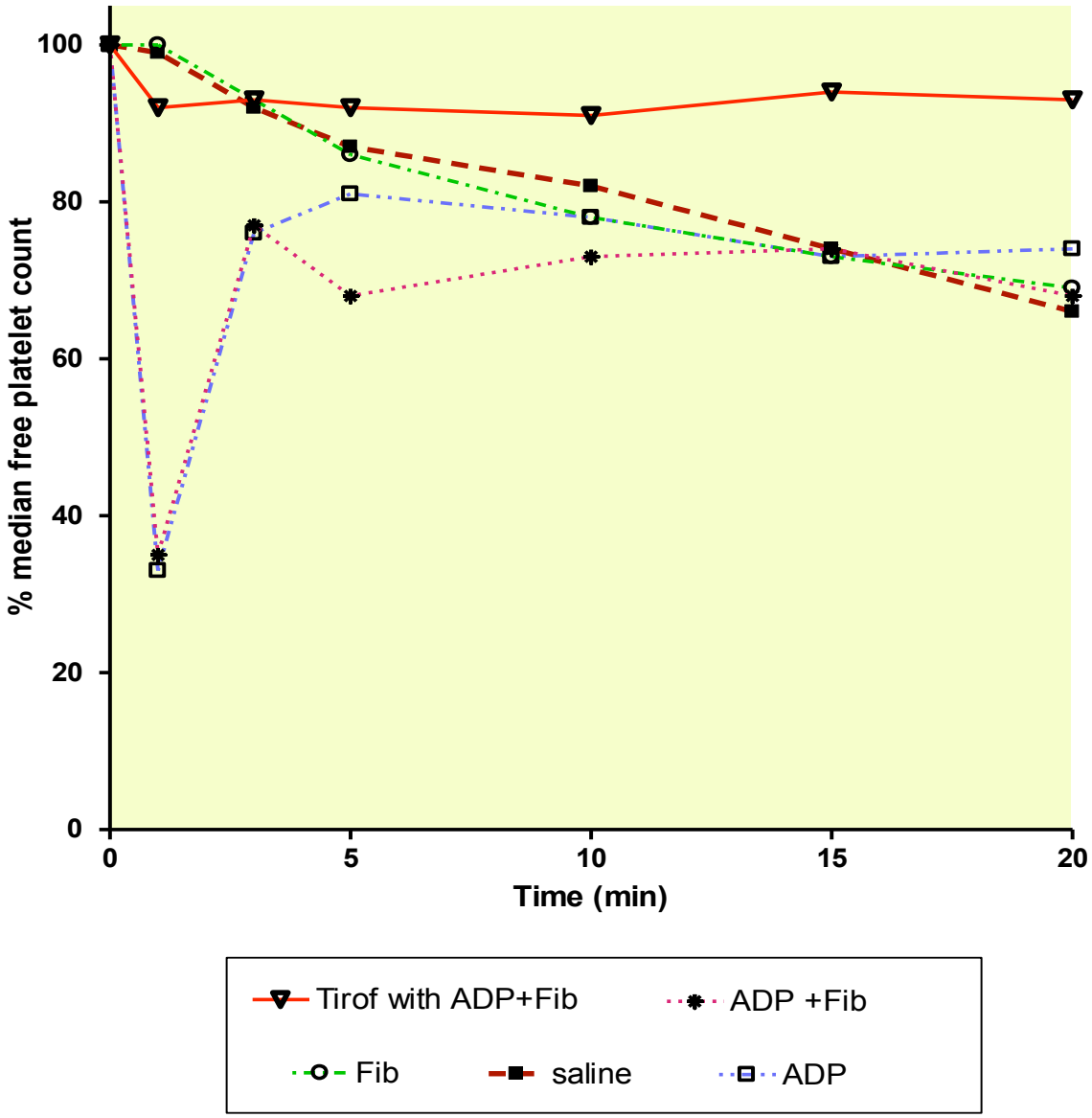

Fig 5.3: The effect of tirofiban on ADP+Fib-induced platelet aggregation

The curves show the effect of ADP (1 µm) only and fibrinogen (0.5 g/l) only on platelet aggregation, in comparison with a combination of ADP (1 µm) together with fibrinogen (0.5 g/l). Then the effect of tirofiban (250 ng/ml) on this combination of ADP plus fibrinogen. Results are expressed as % median free platelet count (n=7).

Table 5.5 The Effect of Tirofiban on 5HT-induced Platelet Aggregation over Time

Time (min)	Saline vs 5HT	5HT vs Tirof + 5HT
1	96 ± 5 vs 82 ± 7 **P = 0.002**	82 ± 7 vs 85 ± 10 **P= NS (0.64)**
3	92 ± 5 vs 75 ± 9 **P = 0.004**	75 ± 9 vs 85 ± 7 **P= 0.02**
5	87 ± 8 vs 71 ± 10 **P = 0.014**	71 ± 10 vs 86 ± 8 **P= 0.01**
10	80 ± 13 vs 66 ± 11 **P = 0.04**	66 ± 11 vs 86 ± 10 **P= 0.02**
15	71 ± 13 vs 63 ± 12 **P= NS (0.18)**	63 ± 12 vs 87 ± 9 **P = 0.006**
20	64 ± 20 vs 60 ± 12 **P= NS (0.56)**	60 ± 12 vs 86 ± 8 **P= 0.005**

Results show the effect of 5HT (5 μM)-induced platelet aggregation compared with saline, and the effect of tirofiban (500 ng/ml) on 5HT (5 μM)-induced platelet aggregation over time. Results are expressed as mean and ± standard deviation and compare percentage free platelet count. 7 healthy volunteers were evaluated.

Notes: serotonin = 5HT; tirof = tirofiban; NS = not significant

5.4.2 Median Platelet Volume Measurement

There was the expected significant increase in MPV (fl) when ADP-induced PSC was compared with saline control (mean and ± SD: 5.79 ± 0.55 vs 6.09 ± 0.55, P = 0.0001, n = 7). Tirofiban (50 – 500 ng/ml) had no significant inhibitory effect on this increase in PSC (6.09 ± 0.55 vs 6.12 ± 0.55, P = NS, n = 7).

The effect of tirofiban on 5HT-induced PSC followed the same trend as above. Thus, 5HT (6.12 ± 0.49 fl) vs saline control (5.79 ± 0.57 fl) demonstrated a significant increase in MPV (P = 0.0004, n = 7). However, tirofiban (50 – 500 ng/ml) did not show any inhibitory effect on this response (6.12 ± 0.49 vs 6.14 ± 0.50, P = NS, n = 7).

The effect of fib (0.5 – 2 g/l) on MPV at various time points (between 1 min and 20 min) was also evaluated. However, there was no apparent change in MPV (n = 7) when compared with saline controls (a representative result from 1 subject is shown below). Thus, MPV at the given time points are illustrated for saline control e.g. 1 min, 6.18 fl; 3 min 6.12 fl; 5 min, 6.18 fl; 10 min, 6.06 fl; 15 min, 6.06 fl and 20 min 6.06 fl. Also the MPV are shown for fib (2 g/l) at the same time points 1min, 6.12 fl; 3 min 6.12 fl; 5 min, 5.99 fl; 10 min, 5.93 fl; 15 min, 6.12 fl and 20 min 6.12 fl. Also, there was no additive MPV effect when fib (0.5 – 1.5 g/l) was used in combination with 5HT (0.25 μM) or ADP (0.2 μM) (results not shown).

5.4 DISCUSSION

PSC is an early phase of platelet activation that precedes platelet aggregation. The technique used in this work to assess PSC is highly sensitive (resolution 0.07 fl) (**Jagroop** *et al.,* 1996; **Jagroop** *et al.,* 2000c; **Jagroop** *et al.,* 2000a; *see Appendix A: PSC reference list with* ***IA Jagroop****)* as compared with others who assessed platelet volume with less sensitive methods (Maurer-Spurej et al., 2001; Wilde *et al.,* 2000). This is why we used our high-resolution technique of measuring MPV to observe if fib can affect PSC. In this study, fib (0.5 – 2 g/l) had no effect on PSC at various time points (between 1 and 20 min) which was in agreement with the findings of others (Negrescu *et al.,* 1995).

Also, ADP and 5HT-induced PSC, was not inhibited by tirofiban (using a range of doses 50 – 500 ng/ml) a potent and specific GPIIb/IIIa receptor antagonist. This work therefore, suggests that fib has no effect on PSC and also that tirofiban does not inhibit PSC. In support of our findings, another study demonstrated that when platelets were pre-treated with EGTA or pre-incubated with RGDS (a fibrinogen receptor antagonist) to prevent the activation of the fibrinogen receptor (the integrin $\alpha_{IIb}\beta_3$), PSC induced by the peptide YFLLRNP still occurred (Negrescu *et al.,* 1995). The technique they used to measure PSC in washed platelets was by recording the light transmission (Negrescu *et al.,* 1995).

In the work carried out here, the technique of measuring PSC is carried out using PRP, and its preparation may influence various aspects of platelet function (Storey *et al.,* 1998). The choice of anti-coagulant is also important. Citrate anti-coagulation was shown to demonstrate falsely high estimates of efficacy of eptifibatide due to the lowering of ionized calcium *in vitro*. Storey et al. also verified that eptifibatide was markedly more effective

both in whole blood and in PRP ($P<0.0005$), when citrate was used as an anti-coagulant rather than hirudin, an anti-coagulant that is a direct thrombin inhibitor and maintains physiological calcium ion levels (Storey *et al.,* 1998)

Platelet agonists (like ADP and 5HT) may amplify platelet activation by binding to specific platelet membrane receptors and signalling the conformational change of GPIIb/IIIa, which then becomes an active receptor for fib initiating aggregation. It would therefore be an advantage to inhibit the effect of fib binding to the GPIIb/IIIa receptor, using a specific antagonist like tirofiban.

This GPIIb/IIIa receptor antagonist reduced the risk of ischaemic complications in patients with unstable angina/non-Q-wave MI in patients undergoing PCI against a background of heparin and aspirin (Song *et al.,* 2007). With this in mind, we observed the effect of tirofiban on fib-induced platelet aggregation. On its own fib showed significant platelet aggregation compared with the saline control. Increased circulating levels of fib, is an emerging predictor of vascular events. Fib levels are raised in patients with vascular disease (Corrado *et al.,* 2010; Stefanadi *et al.,* 2010; Kakafika *et al.,* 2007; Paraskevas *et al.,* 2007). Fib also plays an important role in platelet aggregation by linking activated platelets (Jennings *et al.,* 2009). It is therefore advantageous to inhibit fib-induced platelet aggregation.

GPIIb/IIIa receptor antagonists are very potent inhibitors due to the critical role fib binding plays in platelet aggregation (Huang *et al.,* 2004). When administered intra-venously these drugs are effective as adjuvant therapy in PCI and in ACS (1994; 1997a; 1997b *no authors listed*). The approval of tirofiban covers conservative treatment of MI and

unstable angina, as well as PCI intervention (Bukow et al, 2006). Tirofiban has been shown to safe and effective in patients with acute coronary syndrome. Although, tirofiban when compared with abciximab demonstrates similar efficacy, its clinical use has not been as widespread as initially forecasted (De *et al.,* 2011;Saucedo, 2010;Valgimigli *et al.,* 2010).

In this study, it was shown that tirofiban significantly decreased the aggregating effect of fib. However, it should be noted that GPIIb/IIIa antagonists are qualitatively different from classical anti-platelet agents, such as aspirin or clopidogrel. They do not inhibit platelet activation (i.e. intra-platelet signal generation or conduction) but primarily act outside the platelet by competing with ligand binding that is essential for platelet bridging and aggregate formation.

The concentration of tirofiban used to achieve complete inhibition of fib was 500 ng/ml. Mukherjee *et al.* (Mukherjee *et al.,* 2007) stated that a lower dose of tirofiban (10 μg/kg bolus followed by 0.15 μg/kg/min infusion was inferior to the standard dose of abciximab in patients with PCI. This was possibly due to insufficient platelet inhibition by this low dose. Thus, when a high-bolus dose of tirofiban 25 μg/kg bolus was administered followed by the standard infusion, tirofiban was as effective as abciximab with comparable safety (Mukherjee *et al.,* 2007). Storey *et al.* also showed that higher concentrations (100-1500 nmol/L) of GPIIb/IIa antagonists (eptifibatide, M0852 and GR144053) were required to inhibit platelet aggregation in whole blood compared with PRP turbidimetry (Storey *et al.,* 1998). That is to say that a 3-fold higher concentration of eptifibatide was required to inhibit ADP-induced microaggregate formation (aggregation in whole blood or PRP) than was required to inhibit macroaggregate formation (PRP turbidimetry) (Storey *et al.,* 1998).

This may explain why in my preliminary experiments with agonists-induced aggregation, lower doses (50 ng/ml) of tirofiban, only partially inhibited platelet aggregation. Thus, it was decided that higher concentrations (250-500 ng/ml) of tirofiban should be used. However, tirofiban at this lower concentration (50 ng/ml) did significantly inhibit SPA (table 5.1 & figure 5.1). At the higher concentrations of tirofiban (250-500 ng/ml) we observed complete inhibition of ADP as well as 5HT-induced platelet aggregation, to a level even better than the saline baseline value (table 5.3 and 5.5). In addition, tirofiban was effective in inhibiting platelet aggregation induced with a combination of agonists (ADP plus fib, table 5.4).

Despite being as potent as their intra-venous counterparts, the oral GPIIb/IIIa inhibitors showed no benefit or even increased mortality in clinical trials. Furthermore, most GPIIb/IIIa antagonists have the ability to exert partial agonism. In the presence of high drug levels, this is not a problem, however the low trough levels with oral inhibitors can lead to increased platelet aggregation. There are a number of possible reasons for their failure, including low bioavailability that led to a large peak-trough difference (Cox, 2004). This rational supports the need to achieve higher circulating levels of GPIIb/IIIa. Furthermore, in a clinical setting, despite the administration of GPIIb/IIIa inhibitor the platelets may be 'primed' (since PSC can occur). Therefore, inhibiting PSC (e.g. by administering aspirin, clopidogrel or both drugs together with a GPIIb/IIIa inhibitor) may be relevant. In this context, we previously evaluated the effect of ASA (75 mg/day), clopidogrel (75 mg/day) and both anti-platelet drugs on platelet function in patients with PAD. There was a significant decrease on ADP-induced aggregation after clopidogrel but not after taking ASA.

Dual therapy significantly decreased SPA, which was not significantly altered by either monotherapy. The same phenomenon was observed with 5HT-induced aggregation (**Jagroop** *et al.,* 2004).

The risk/benefit ratio of GPIIb/IIIa antagonists may be due to its differential effects on microaggregation and macroaggregation. It appears that it may be easier to inhibit the formation of larger aggregates of platelets (macroaggregates) than to prevent pairing of platelets and formation of smaller aggregates (micro-aggregates) (Storey *et al.,* 1998). The hypothesis is that different GPIIb/IIIa antagonists will have different ratios of affinities for the resting receptor and the activated receptor. Also, adhesive proteins (other than fib) may play an important role in macroaggregation and finally a variable inhibition of the release reaction by GPIIb/IIIa antagonists as a result of outside-in signaling may have a greater effect on *in vitro* macroaggregation. Moreover, the differences in the outcome of studies looking at the effect of GPIIb/IIIa antagonists on the incidence of death and MI, may relate to the levels of these drugs on inhibition of microaggregation (Storey *et al.,* 1998).

In these studies, whole blood platelet aggregation was used, as it offers rapid results without imposing too many changes on the platelet population. Others have assessed platelet aggregation using PRP. There is however, some controversy as to whether PRP should be platelet count adjusted using PPP or if it is better to use native PRP. Moreover, the adjustment of PRP using PPP may falsify results. PPP is prepared by vigorous centrifugation where physiological agonists are released from platelets (remaining after removal of the PRP) activated by the high shear stress. Furthermore, even the 'slower' centrifugation for PRP preparation can activate platelets. Thus, adjusting PRP with this PPP may affect platelet

aggregation measurements. In contrast, *Mani H et al.* have demonstrated that there were no significant differences in the maximum platelet aggregation response when using either native PRP or adjusted PRP from healthy subjects and patients on anti-platelet treatment (Mani *et al.,* 2005). In fact, the variation in the results was much larger when adjusted PRP was used. Moreover, in another study (Linnemann *et al.,* 2008) where healthy subjects were compared with patients on anti-platelet therapy, the variability of aggregation in adjusted PRP in the subgroup of healthy subjects ranged from 9.2 - 95.3% (5^{th} to 95^{th} percentile). This is relative to 77.6 - 95.5% in the non-adjusted PRP when determining aggregation to arachidonic acid 0.5 mg/ml. Thus, the time consuming process of PRP adjustment may not be necessary for platelet aggregation measurements.

From this study, it is concluded that tirofiban significantly inhibited agonists (ADP, 5HT)-induced platelet aggregation. This GPIIb/IIIa receptor antagonist is effective in inhibiting SPA as well as platelet aggregation induced by a combination of agonists (fib and ADP). That is to say, that tirofiban appears to be non-specific as it was effective in inhibiting aggregation (a later phase of platelet activation) induced by not only fib but also other agonists used alone or even in combination. In addition, it was effective in reducing even a very high concentration of fib (8 g/l, results not shown). We have also shown that tirofiban does not have an effect on PSC an early phase of platelet activation. (**Jagroop** *et al.,* 2008).

It is possible that in a clinical setting, despite the administration of a GPIIb/IIIa inhibitor the platelets may be 'primed' (at PSC) therefore maintaining a high degree of inhibition of the later stages of platelet activation may be very relevant. Further studies are

needed to reconfirm the findings, where fib does not affect PSC, and that in this model tirofiban does not inhibit agonist-induced PSC. It is difficult to say exactly what the clinical relevance of this is.

5.5 ACKNOWLEDGEMENTS

My thanks to thank Merk Sharp and Dohme, Hertfordshire, UK., who kindly donated tirofiban for use in these experiments.

CHAPTER 6

A New Rapid Method to Measure Human Platelet Cholesterol: A Pilot Study

6.1 (i) AIMS, BACKGROUND AND HYPOTHESIS

Aims: The aim of this study was to develop a rapid, safe, reproducible and simple method to measure platelet cholesterol (PC). I also aimed to determine if PC correlated with circulating lipid levels.

Background: Platelet hyperactivity has been linked to increased PC. The rational is that a rise in PC may cause increased platelet responses since cholesterol affects platelet membrane fluidity. Increased PC may also modulate the conformational state of platelet receptors, thereby indirectly affecting the effectiveness of antiplatelet drugs. Moreover, PC can be used as a marker of 'tissue' cholesterol. It is however, impractical to measure 'tissue' cholesterol, but, platelets are easily accessible. So far, the methods used to assess PC were too cumbersome. They involved a complex extraction process with the use of toxic and potentially harmful chemicals and an overnight incubation, making it time consuming and thus not suitable for serial measurements. Using the more complex method to measure PC, others have shown that lovastatin decreased plasma and PC levels and normalised elevated platelet fluidity and aggregation in hypercholesterolaemic patients. Thus, the development of a simpler and rapid method to measure PC would make studies like this easier to perform. It would be of interest to see if PC is affected before and after treatment with statins in hypercholesterolaemic patients, using my new method to assess PC.

Hypothesis: The hypothesis is that using a rapid PC method we will be able to evaluate platelet receptor function (e.g. $P2Y_1$, $P2Y_{12}$ and GPIIb/IIIa) before and after treatment with statins. We will then be able to relate these findings to PC levels.

6.1 (ii) INTRODUCTION

Platelets have been implicated in the pathogenesis of cardiovascular disorders, including unstable angina, stroke, myocardial infarction and sudden cardiac death (Alexander *et al.,* 2010; Garg *et al.,* 2010; Sakhuja *et al.,* 2010). We suggest that platelet cholesterol (PC) may be another indicator of "tissue" cholesterol status in patients with vascular disease. However, the methods available so far to measure PC, involve a complex extraction process and are time consuming and therefore not suitable for multiple measurements. There are also safety issues since this process involves the use of flammable and potentially harmful chemicals (like chloroform and methanol) (Biro *et al.,* 2005; Bligh *et al.,* 1959; Garcia-Guerra *et al.,* 1998). For example, a procedure for the isolation of glycoproteins from human platelet plasma membrane involves the differential extraction of platelet membranes using Triton-X, SDS solubilizaton of the 4% Triton-X supernatant, zonal centrifugation in a sucrose density gradient, and finally the preparative high-performance size exclusion chromatography. It should also be noted that Triton-X is damaging as well as dangerous to the environment. Moreover, Triton-X may contain dioxane which is a probable carcinogen, toxic and harmful by inhalation (Biro *et al.,* 2005; Bligh *et al.,* 1959; Garcia-Guerra *et al.,* 1998). Ethylene oxide may also be present in Triton-X, this too is a carcinogen, mutagen and reproductive hazard. Even moderate levels (</> 0.1%) may rapidly be fatal if inhaled (Bligh *et al.,* 1959; Poss *et al.,* 2003). Therefore, developing a simpler and safer method to measure PC is important, especially if large numbers of samples need to be analyzed.

In the present study, it was investigated if there a correlation between PC and circulating low density lipoprotein cholesterol (LDL-C) levels exists. LDL-C affects the function of blood cells, which play an important role in atherosclerosis and its complications (Steinbrecher *et al.,* 1990). For example, LDL-C may influence the aggregation of isolated platelets (Freire de *et al.,* 2007; Naseem *et al.,* 1997; Vlasova, 2000). Platelets possess receptors for plasma lipoproteins (oxidised and native LDL-C) (Bochkov *et al.,* 1995; Pedreno *et al.,* 1994) and the binding of LDL-C to these receptors induces a cascade of intra-cellular biochemical reactions resulting in platelet activation (Block *et al.,* 1988;Bruckdorfer, 1989). Moreover, the lipid composition of platelets membranes is known to affect platelet activity (Kitagawa *et al.,* 1985). It has been shown that an excess in PC results in platelet hyperactivity, and that there is a correlation with increased levels of PC and a decrease in plasma membrane fluidity (Padmavathi *et al.,* 2010; Shattil *et al.,* 1976).

The aim of the study in this chapter, was to develop a simplified method to measure PC and assess if there is a correlation between PC and LDL-C, high-density lipoprotein (HDL-C), LDL-C/HDL-C ratio, triglycerides (TG), non-HDL-C (TC minus HDL-C) and serum total cholesterol TC.

6.2 METHODS

6.2.1 Selection of Subjects and Collection of Blood

The main study population consisted of 47 healthy subjects (23 male; 24 female; mean age 37 ± 9 years). No drugs affecting platelet function were taken in the 8 days preceding blood sampling. All subjects participated after giving their informed consent. The study was approved by the University College London Ethics Committee. Whole blood was drawn by venepuncture after an overnight fast. Blood was obtained from an antecubital vein using a 21G needle with minimum stasis (as described in Chap 2).

6.2.2 Serum Lipid Analysis

For the determination of serum lipid analysis, 5 ml blood was collected in a serum gel tube, allowed to clot and then centrifuged (2,000 x g, 15 min at 4^0C) collecting the supernatant for analysis of serum TC, LDL-C, TG and HDL-C using a 'Roche Modular P' system and the associated kits (Roche Diagnostics, Indianapolis, USA). All our lipid assays participate in The United Kingdom National External Quality Assessment Service (UK NEQAS) which provides a comprehensive worldwide service that enables laboratories to fulfil quality goals. UK NEQAS comprises a network of schemes operating from a number of centers based at major hospitals, research institutions and universities throughout the UK. The services cover qualitative and interpretative investigations in clinical chemistry along with a variety of other disciplines.

6.2.2.1 Serum HDL-C Measurement

In this study, an automated method for the direct determination of serum HDL-C based on polyethylene glycol (PEG)-modified enzymes and dextran sulphate, was used. When cholesterol esterase and cholesterol oxidase enzymes were modified by PEG, they showed selective catalytic activities towards lipoprotein fractions, with the activity increasing in the order: LDL-C < very low density lipoprotein cholesterol = chylomicrons < HDL-C (Friedewald *et al.,* 1972).

In the presence of Mg^{2+} ions, dextran sulphate selectively formed water soluble complexes with LDL-C, VLDL and chylomicrons which were resistant to PEG-modified enzymes. The cholesterol concentration of HDL-C was determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups. Cholesterol esters were broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol was oxidized to Δ^4-cholestenone and hydrogen peroxide. With the presence of peroxidase, the hydrogen peroxide generated reacted with 4-amino-anti-pyrine and HSDA (sodium N-(2-hydroxy-3-sulfopropyl)-3,-5-dimethoxyaniline and formed a purple-blue dye. The colour intensity of this dye was directly proportional to the cholesterol concentration and was measured photometrically (Roche Modular analyzer).

6.2.2.2 Serum TG Measurement

TG was determined by a method based on using a lipoprotein lipase from microorganisms for the rapid and complete hydrolysis of triglycerides to glycerol, followed

by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacted with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase and formed a red dyestuff. The colour intensity of the red dyestuff formed was directionally proportional to the triglyceride concentration and was measured photometrically (Roche Modular analyzer).

6.2.2.3 TC Measurement

The method to measure serum TC was based on the determination of Δ^{4-} cholestenone after enzymatic cleavage of the ester by cholesterol esterase, conversion of cholesterol oxidase, and subsequent measurement by the Trinder reaction of the hydrogen peroxide formed. Optimisation of the ester cleavage (>99.5%), allowed standardisation with primary and secondary standards and a direct comparison with reference methods. Cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. Cholesterol esters were cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol was converted by oxygen by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide created formed a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The colour intensity was directionally proportional to the concentration of cholesterol and was determined photometrically (Roche Modular analyzer) (test range 0.08 to 20.7 mmol/l).

6.2.2.4 Serum LDL-C Measurement

LDL-C was estimated by the Friedewald equation (Friedewald *et al.,* 1972):

$$LDL\text{-}C = TC - HDL\text{-}C - TG\ (mmol/l)/2.2$$

This equation (Friedewald *et al.,* 1972) is not valid at TG concentrations (>4.5 mmol/l) or non-fasting samples containing chylomicrons.

6.2.3 Blood Collection and Platelet Preparation for PC Measurement

A further 9 ml of blood was also collected in polystyrene tubes containing 3.8% tri-sodium citrate (9 parts blood to 1 part citrate) for the analysis of PC. This citrated sample was gently inverted to allow mixing without disrupting the platelets. It was then centrifuged at x150 g for 15 min (IEC Centra-7R refrigerated centrifuge; International equipment company, USA) to prepare platelet rich plasma (PRP). PRP was separated using a plastic Pasteur pipette to gently aspirate the top layer avoiding the 'buffycoat' and the red cells (see chapter 2, general methods). The PRP volume (ml) and count (10^9/L) was measured (Coulter Counter Act diff) and recorded as this result was needed for the final calculation of the PC. The PRP was placed into 'conical bottomed' plastic tubes and again centrifuged at 300 g for 30 min at 4^0C. The platelet poor plasma (PPP) was poured off leaving a platelet pellet which was resuspended in 1 ml saline and then transferred to an Eppendorf tube.

Platelet membrane disruption was achieved in 2 steps. Firstly, the 1 ml saline solution containing the platelet pellet was put through 2 freeze (at -20^0C) and thaw (room temperature) cycles. Then it was re-suspended using a whirlimixer. The Eppendorf tube

containing the 're-suspended pellet' was then kept on crushed ice (to prevent the sample from heating up) and sonicated for 3 cycles, each for 10 sec, at 30 amplitude microns using a probe Sonicator (M.S.E. Soniprep, Crawly, UK). This was based on previous experiments where platelet membrane disruption was achieved to allow 5HT release (Fawcett and **Jagroop** *et al.,* 1998; Frampton and **Jagroop** *et al.,* 2006). It was important to resuspend the pellet in the smallest possible volume of saline that would allow a sonication process, but will also be concentrated enough to allow platelet cholesterol measurement. During this sonication process care was taken to prevent 'froth' formation but to still obtain a 'clear pellet solution' (Fawcett and **Jagroop** *et al.,* 1998; Frampton and **Jagroop** *et al.,* 2006) that could be used for the analysis PC.

6.2.3.1 PC Measurement

Platelet cholesterol (PC) measurement was based on an enzymatic colorimetric test. The clear pellet solution (as prepared above) was added to a solution 'R1' (CHOL, cholesterol CHOD-PAP; Roche Diagnostics, Indianapolis, IN, USA). Cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. Cholesterol esters were cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol was converted by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide created formed a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The color intensity was directly proportional to the concentration of cholesterol and was determined photometrically (Roche Modular P system).

6.2.4 Repeatability

To determine the repeatability (i.e. corresponding to within-run precision) of this PC method, 3 healthy subjects were selected (2 males, 1 female). Blood was collected and prepared for PC measurement (as described below). 20 successive PC measurements were carried out under the same conditions on the same day.

6.2.5 Reproducibility

To determine the closeness of agreement between results of measurements performed under changed conditions, in this case, time (i.e. corresponding to between-run precision), 2 subjects were selected (1 male and 1 female). Blood was collected and prepared for PC measurement on 8 different days over a period of 2 months.

6.2.6 Statistical Analysis

Results are expressed as mean and ± SD. Correlations were assessed using non-parametric tests (Spearman, correlation coefficient). Significance was considered as $P<0.05$ (two-tailed).

6.3 RESULTS

6.3.1 Repeatability of PC method

The within-run precision of PC carried out on 3 subjects (20 times each) showed a mean coefficient of variation (CV) of 4.8% (4.2, 4.6 and 5.5%, respectively, for each subject).

6.3.2 Reproducibility of PC method

The between-run precision of PC was carried out on 2 subjects and assessed on 8 different days over 2 months. For subject 1, the PC method measured a CV of 8.9 % with a mean of 426 ± 38 μmol/10^{12} platelets. The CV of LDL-C was 7.5% with a mean of 3.5 ± 0.3 mmol/l. For subject 2, the PC method showed a CV of 8.1% with a mean of 371 ± 30 μmol/10^{12} platelets. For the LDL-C the CV was 5.6% with a mean of 3.6 ± 0.2 mmol/l.

6.3.3 The Reportable Range PC Method

The range observed with our PC method was between 173 – 830 μmol/10^{12} platelets.

6.3.4 Study Population Selection

The study population (n = 47) was split into 2 groups: Group 1: LDL-C < 2.6 mmol/l (n = 22) and Group 2: LDL-C > 2.6 mmol/l (n = 25). These LDL-C limits are those recommended by the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) (Grundy *et al.,* 2004;Smith, Jr. *et al.,* 2006)

Table 6.1 The Lipid Profiles of all Subjects, Categorized into 2 Groups

	LDL-C < 2.6 (mmol/l) (Group 1, n=22) Mean ± SD	LDL-C > 2.6 (mmol/l) (Group 2, n=25) Mean ± SD
LDL-C	1.93 ± 0.36	3.61 ± 0.73
HDL-C	1.64 ± 0.45	1.33 ± 0.49
LDL-C/HDL-C	1.24 ± 0.54	2.89 ± 1.29
TG	1.18 ± 0.77	1.44 ± 0.67
non-HDL-C	2.51 ± 0.48	4.32 ± 0.85
TC	4.15 ± 0.48	5.65 ± 0.81

Results are expressed as mean ± SD for LDL-C (mmol/l); HDL-C (mmol/l); LDL-C/HDL-C ratio; TG (mmol/l); non-HDL-C (mmol/l) and TC (mmol/l). Subjects with LDL-C <2.6 mmol/l (Group 1, n = 22; 6 male; 16 female; mean age 34 years) as well as subjects with LDL-C > 2.6 mmol/l (Group 2, n=25; 17 male; 8 female; mean age 39 years) are listed.

LDL-C = low density lipoprotein cholesterol (mmol/l)
HDL-C = high density lipoprotein (mmol/l)
TG = triglycerides (mmol/l)
TC = total cholesterol (mmol/l)

6.3.4.1 Group 1 (LDL-C < 2.6 mmol/l)

In Group 1 (LDL-C < 2.6 mmol/l, n = 22, 6 male, 16 female, mean age 34 years) the mean PC was 412 ± 147 μmol/10^{12} platelets with a median and of 388 (173 – 830 μmol/10^{12} platelets). There was no significant correlation between PC and LDL-C (1.93 ± 0.36 mmol/l), HDL-C (1.64 ± 0.45 mmol/l), LDL-C/HDL-C (1.24 ± 0.54 mmol/l), TG (1.18 ± 0.77 mmol/l), non-HDL-C (2.51 ± 0.48 mmol/l) or TC (4.15 ± 0.48 mmol/l). The mean value for lipid variables were: LDL-C 1.93 ± 0.36, HDL-C 1.64 ± 0.45, LDL-C/HDL-C 1.24 ± 0.54, TG 1.18 ± 0.77, non-HDL-C 2.51 ± 0.48 and TC 4.15 ± 0.48 mmol/l.

6.3.4.2. Group 2 (LDL-C > 2.6 mmol/l)

In group 2, (LDL-C > 2.6 mmol/l, n = 25, 17 male, 8 female, mean age 39 years) the mean PC was 377 ± 120 μmol/10^{12} platelets. There was a positive correlation (r_s = 0.45, P = 0.02) between PC and LDL-C. There was also a positive correlation between PC and LDL-C/HDL-C (r_s = 0.45; P = 0.02), PC and TG (r_s = 0.43, P = 0.03) and PC and non-HDL-C (r_s = 0.53, P = 0.007). There was no significant correlation between PC and HDL-C (r_s = -0.31) or between PC and TC (r_s = 0.34). These results are illustrated in figures 6.1-6.4). The mean value for lipid variables were: LDL-C 3.61 ± 0.73, HDL-C 1.33 ± 0.49, LDL-C/HDL-C 2.89 ± 1.29, TG 1.44 ± 0.67, non-HDL-C 4.32 ± 0.85 and TC 5.65 ± 0.81 mmol/l.

Table 6.2 Statistical Analysis for Subjects with LDL-C > 2.6 mmol/l

	Spearman r_s	**P**
PC vs LDL-C	0.45	**0.02**
PC vs LDL-C/HDL-C	0.45	**0.02**
PC vs TG	0.43	**0.03**
PC vs non-HDL-C	0.53	**0.007**
PC vs HDL-C	-0.31	**NS (0.13)**
PC vs TC	0.34	**NS (0.09)**

Results show statistical analysis (non-parametric correlation, Spearman, two-tailed) for subjects with LDL-C > 2.6 mmol/l (n=25). Correlations were carried out for PC (μmol/10^{12} platelets) **with** LDL-C (mmol/l); LDL-C/HDL-C ratio; TG (mmol/l); non-HDL-C (mmol/l) and with HDL-C (mmol/l) and TC (mmol/l).

Platelet cholesterol (PC, μmol/10^{12} platelets)

LDL-C = low density lipoprotein cholesterol (mmol/l)

HDL-C = high density lipoprotein (mmol/l)

TG = triglycerides (mmol/l)

TC = total cholesterol (mmol/l)

ns = non significant

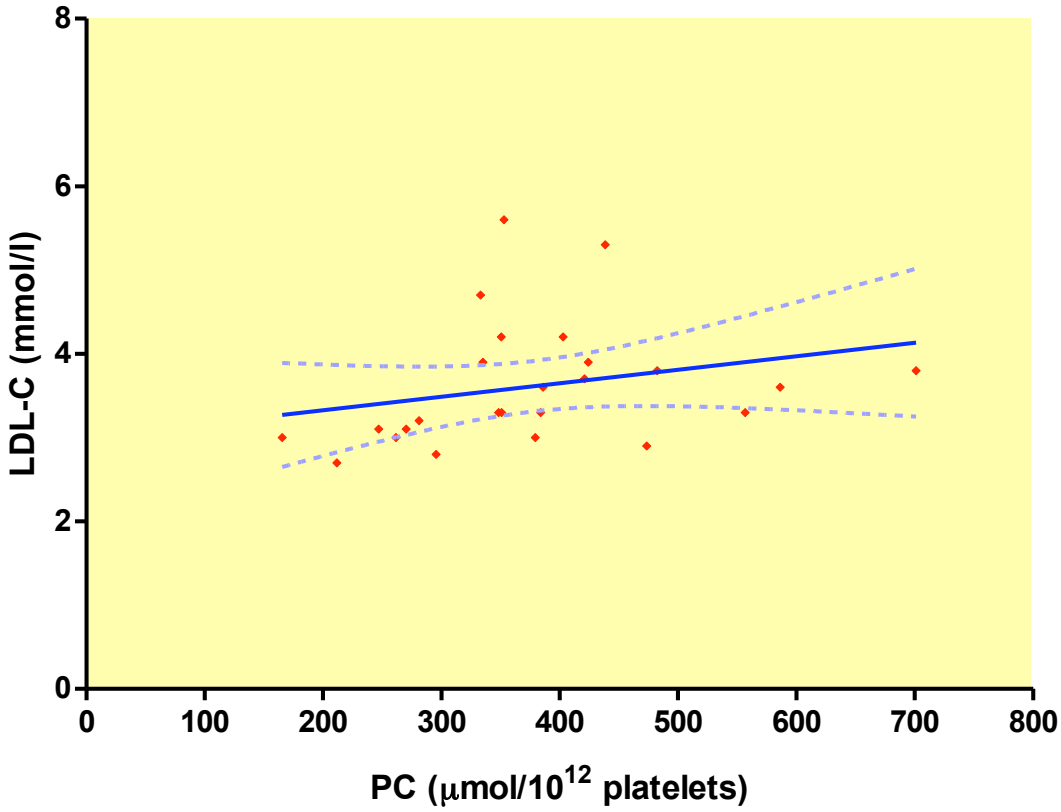

Figure 6.1 The relationship between PC and LDL-C

The curve shows the relationship between platelet cholesterol (PC, μmol/10^{12} platelets) and low density lipoprotein cholesterol (LDL-C, mmol/l). Spearman correlation coefficient = 0.45; P = 0.02; n = 25. The dashed lines represent 95% Confidence Intervals (CI).

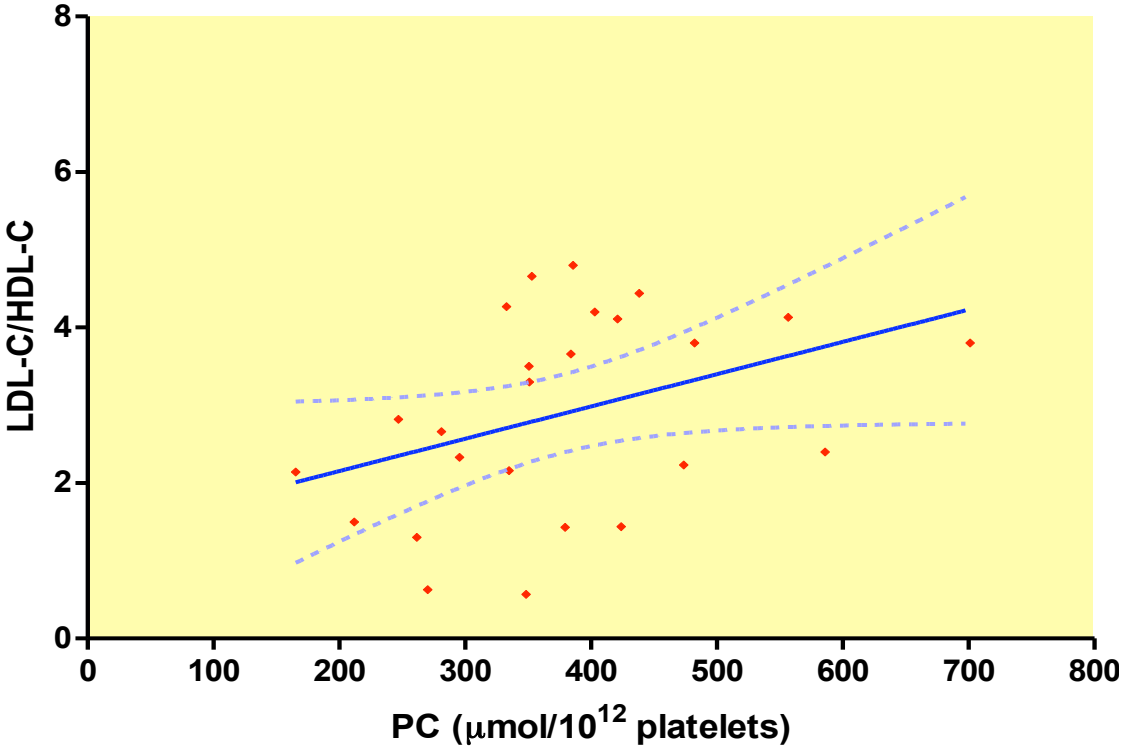

Figure 6.2 The relationship between PC and LDL-C/HDL-C ratio

The curve shows the relationship between platelet cholesterol (PC, μmol/10^{12} platelets) and low density lipoprotein cholesterol/high density lipoprotein cholesterol ratio (LDL-C/HDL-C). Spearman correlation coefficient = 0.45; *P = 0.02;* n = 25. The dashed lines represent 95% Confidence Intervals (CI).

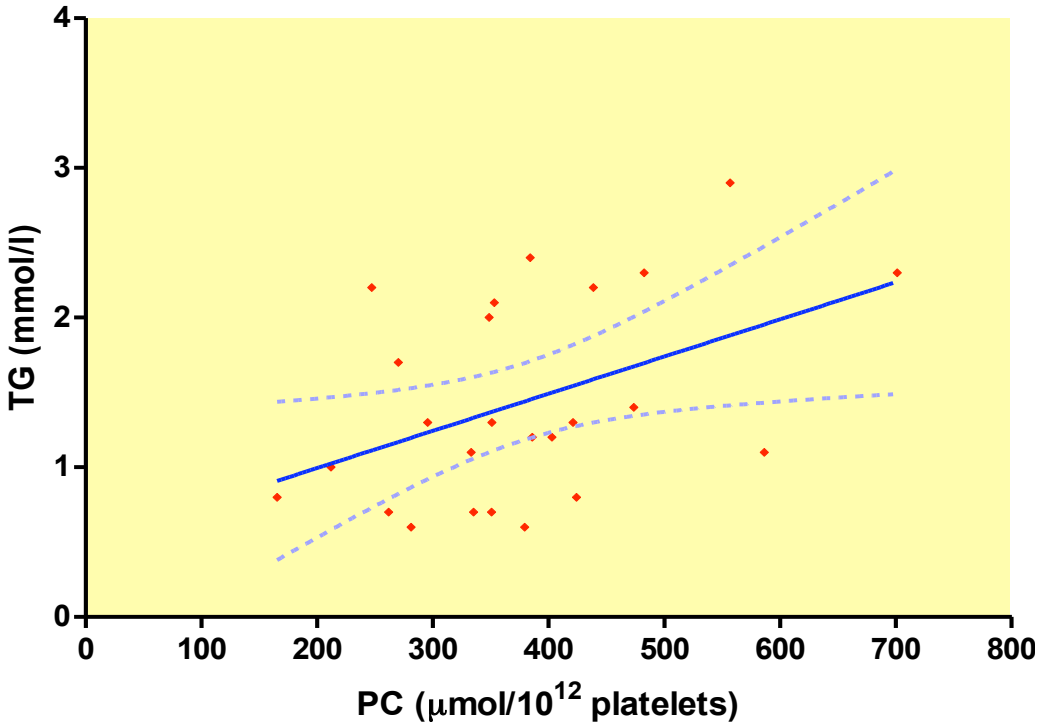

Figure 6.3 The relationship betwen PC (µmol/10^{12} platelets) and TG (mmol/l)

The curve shows the relationship between platelet cholesterol (PC, µmol/10^{12} platelets) and Triglycerides (TG, mmol/l). Spearman correlation coefficient = 0.43; P = 0.03; n = 25. The dashed lines represent 95% Confidence Intervals (CI).

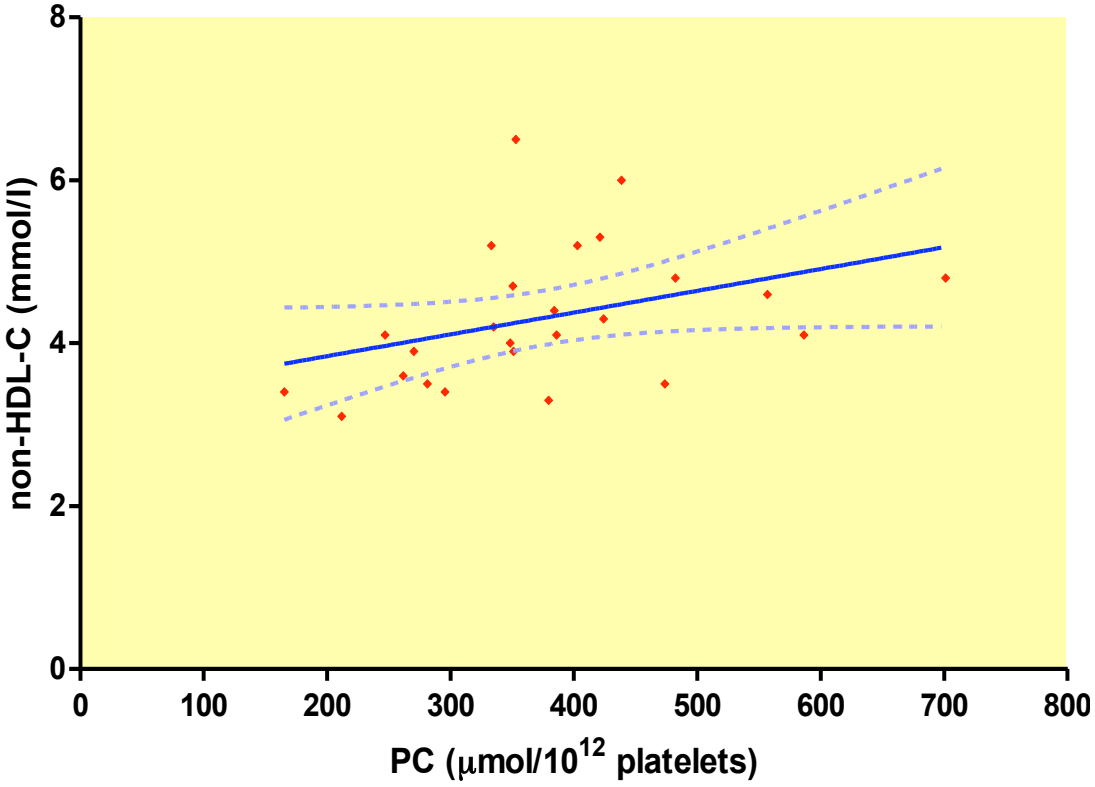

Figure 6.4 Relationship between PC (μmol/10^{12} platelets) and non-HDL-C (mmol/l)

The curve shows the relationship between platelet cholesterol (PC μmol/10^{12} platelets) and non-high density lipoprotein cholesterol (non-HDL-C, mmol/l). Spearman correlation coefficient = 0.53; *P = 0.007;* n=25. The dashed lines represent 95% Confidence Intervals (CI).

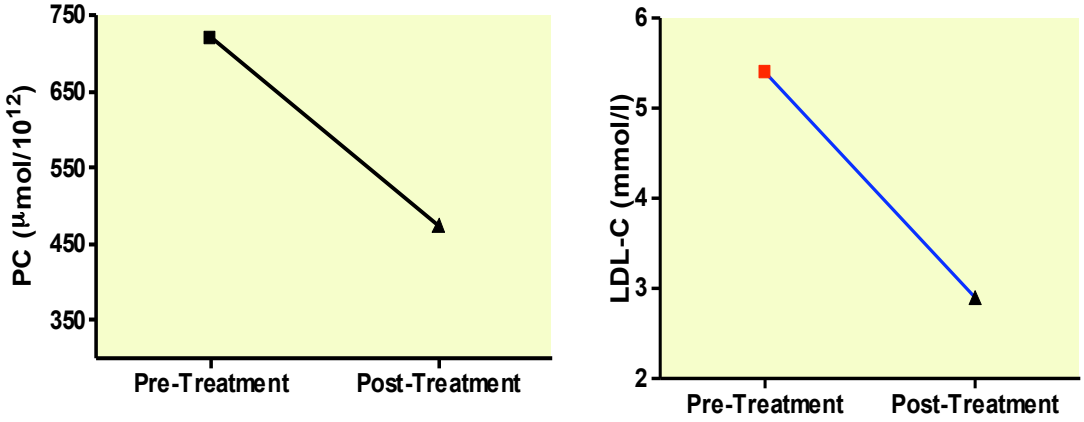

Figure.6.5: Pre- and Post-drug treatment of PC and LDL-C.

The graphs show pre- and post-drug treatment (thyroid hormone therapy) of platelet cholesterol (PC, µmol/10^{12} platelets) and low-density-lipoprotein-cholesterol (LDL-C,mmol/l) in a hypothroid subject. PC was decreased by 34.2% (720 to 474mmol/10^{12} platelets) and LDL-C was decreased by 68.9% (5.4 to 2.9 mmol/l) after six months of drug therapy.

6.3.5 Pre- and Post-thyroid Hormone Replacement Treatment

The results of the lipid profile 1 subject we tested indicated that he was hypothyroid. His pre-treatment LDL-C, non-HDL-C and PC were 5.4 mmol/l, 6.1 mmol/l and 720 µmol/10^{12} platelets, respectively. Subsequently, he was treated with thyroid hormone replacement and became euthyroid. The corresponding post-treatment values (6 months) were LDL-C 2.9 mmol/l, non-HDL-C 1.9 mmol/l, and PC 474 µmol/10^{12} platelets, respectively (see figure 6.5).

DISCUSSION

In this work, a simplified and less time-consuming method for the measurement of PC was developed. This method is more suited to multiple measurements, since it does not involve complicated extraction stages, as was previously used in other techniques (Day *et al.,* 1997;Padmavathi *et al.,* 2010; Vevera *et al.,* 2005). The PC levels we obtained using our technique were in agreement with those reported by others (Blache *et al.,* 1995; Chetty *et al.,* 1992; Hochgraf *et al.,* 1994; Rabini *et al.,* 1995; Sato *et al.,* 1990). However, this method needs to be further assessed with a larger number of samples. In addition, it was observed, that it is limited by not being sensitive at lower LDL-C levels (i.e. <2.6 mmol/l). It is proposed that this method is evaluated in patients before and after treatment with statins.

This work suggest, that PC could be used as a marker to assess "tissue" cholesterol status of patients with vascular disease. These patients tend to be on cholesterol lowering drugs (e.g. statins) that may have an effect on PC. Using an extraction method, *Day et al* demonstrated that cell membrane cholesterol significantly decreased (4.19 to 3.52 fmol/cell) after treating 12 hypercholesterolaemic patients for 6 weeks with simvastatin (20 mg/day) (Day *et al.,* 1997). Moreover, a significant decrease in serum cholesterol (6.57 ± 1.26 mmol/l to 5.10 ± 0.42 mmol/l, $P<0.0001$) and a non-significant decrease (0.755 ± 0.115 to 0.688 ± 0.098, $P = 0.17$) in the ratio of cholesterol/total phospholipids (CH/PL) were found after 2 months of simvastatin (20 mg/day) therapy with 17 hypercholesterolemic patients (Vevera *et al.,* 2005).

Although statins are not considered as anti-thrombotic drugs, they exert other beneficial effects along with improving the lipid profile. These 'pleiotropic' actions include improved endothelial function, reduced oxidative stress and less platelet adhesion (Ludman *et al.,* 2009). This may be a reason for the improvement in cardiovascular risk seen in clinical trials is incompletely explained by cholesterol lowering alone. Therefore it has been suggested, that the benefit from lipid lowering drugs may also involve non-lipid mechanisms. For example, beneficial effects on the arterial wall, improved endothelial function, and a positive effect on blood rheology and thrombogenesis (Milionis *et al.,* 2003; Saw *et al.,* 2009).

It is well documented that increased platelet activity is associated with hyperlipidemia (Chetty *et al.,* 1992; Labios *et al.,* 2005). However, few studies have addressed platelet activation and hypercholesterolemia and the potential effect of lipid lowering drugs on platelet activity. One group studied the effect of atorvastatin (20 mg/day) on platelet activation in hypercholesterolemic patients and found that after treatment, platelet function was normalized and there was a reduced GPIIb/IIIa response to ADP (Labios *et al.,* 2005). These findings are in agreement with others who showed that platelets from hyperlipidaemic patients are hypersensitive to aggregation, with greater thromboxane B_2 (TXB_2; the spontaneous breakdown product of TXA_2) production and 5HT release induced by thrombin when compared with platelets from normolipidemic controls (Chetty *et al.,* 1992).

These findings show that this PC method is reproducible and that PC levels were significantly correlated with serum LDL-C in subjects whose LDL-C was > 2.6 mmol/l.

Furthermore, significant correlations were also found for LDL-C/HDL-C ratio, TG, and non-HDL-C. It is of interest that the strongest correlation was with non-HDL-C, which in some circumstances is superior to LDL-C in predicting cardiovascular risk (Hoenig, 2008). These findings are in agreement with others who demonstrated a significantly higher platelet cholesterol ester in hyperlipidaemic patients (with LDL-C 5.72 ± 1.16) (Chetty *et al.,* 1992). Their results also extended other findings where the levels of cholesterol and total phospholipids in type II hypercholesterolemia were 80% and 60% higher, respectively in platelet membranes of hypercholesterolemic subjects compared with their control group (Mosconi *et al.,* 1988).

We suggest that the increase in PC content may be sufficient to cause the increase platelet responses observed by others (Chetty *et al.,* 1992), since cholesterol affects platelet membrane fluidity (Shattil *et al.,* 1978). Membrane lipid fluidity is a measure of the dynamic state of the membrane and is a an important determinant of cell function (Shinitzky, 1984;Spector *et al.,* 1985). Moreover, lovastatin decreases plasma and PC levels and normalises elevated platelet fluidity and aggregation in hypercholesterolemic patients. Also, platelets from hypercholesterolemic patients had a higher molar ratio of cholesterol to phospholipids (Hochgraf *et al.,* 1994). This corroborates with the findings of others who also demonstrated similar increased molar ratio of cholesterol to phospholipids in hypercholesterolemic patients (Shattil *et al.,* 1975; Shattil *et al.,* 1976).

In this study, the method of measuring PC has a disadvantage of not being sensitive if subjects have a lower circulating level of LDL-C (mean ± SD 1.93 ± 0.36 mmol/l). However, these levels of LDL-C are unlikely to require lipid lowering treatment. This

method also needs to be evaluated in larger numbers of subjects/patients before and after the use of lipid lowering drugs (e.g. statins).

It is of interest that during our study one subject was found to be hypothyroid. Subsequently, he was treated with thyroid hormone replacement and became euthyroid. His pre-treatment LDL-C, non-HDL-C and PC were 5.4 mmol/l, 6.1 mmol/l and 720 μmol/10^{12} platelets, respectively. Corresponding post-treatment values (6 months) were 2.9 mmol/l, 1.9 mmol/l, and 474 μmol/10^{12} platelets, respectively. Thus, there was a decrease by 46.3% in LDL-C, 68.9% in non-HDL-C and 34.2% in PC (see figurer 6.5).

In conclusion, this new technique of measuring PC has the advantage over previously used methods in that it is reproducible, rapid, safe and simple. Thus, it may be useful for multiple sampling when investigating changes in PC in hypercholesterolaemic patients. Whether any difference in PC represents changes in other tissues remains to be established.

CHAPTER 7

General Discussion and

Suggestions for Future Work

GENERAL DISCUSSION

Others have previously shown that clopidogrel inhibits platelet aggregation, but the work presented in this thesis is the first report showing that this anti-platelet agent also affects platelet shape change (PSC), an early phase of platelet activation. Clopidogrel administration in patients with peripheral arterial disease (PAD) inhibited ADP-induced PSC. This finding might be expected since clopidogrel acts on the platelet ADP receptor ($P2Y_{12}$). In addition, the fact that clopidogrel, also inhibited 5HT -induced PSC is interesting. Furthermore, there was a more significant inhibition of ADP- and 5HT-induced PSC with clopidogrel than with aspirin. It is unclear as to why PSC was induced by non-ADP (i.e. 5HT) agonist was inhibited by clopidogrel. This effect may be attributed to a general decrease in platelet activity ('resting state'). Others have also shown inhibition of platelet responses to non-ADP agonists (e.g. 5HT) after administering clopidogrel to dogs (Yao *et al.,* 1992). These effects of clopidogrel on early stages of platelet activation may partly account for its efficacy in preventing thrombosis.

With the use of a high-resolution channelyzer, it was possible to obtain an IC_{50} for the $P2Y_1$ receptor antagonist (MRS 2179) since we could achieve up to 100% inhibition of PSC. Thus, using this technique, there is the potential to determine the potency of other purinoceptor inhibitors. Others showed that the $P2Y_1$ receptor is essential for platelet aggregation, but an IC_{50} has not been previously derived for a $P2Y_1$ receptor-blocker using PSC in human platelets. In contrast to the $P2Y_1$ receptor, the $P2Y_{12}$ receptor antagonist used provided only reversible and sub-maximal inhibition of PSC. Thus, we support others

(Gachet, 2001a; Gachet, 2005; Lubbe *et al.,* 2002) who had suggested that antagonists to the $P2Y_1$ receptor may become useful anti-platelet drugs. It is not possible to evaluate the IC_{50} of clopidogrel *in vitro* because this is a pro-drug that has to be metabolized by the liver to produce an active a $P2Y_{12}$ receptor antagonist.

Tirofiban, added *in vitro*, had no inhibitory effect on either ADP- or 5HT-induced PSC. We experimented with 'low to high' (50-500 ng/ml) doses of tirofiban to ensure that there was no inhibitory effect. The fact that tirofiban, a potent and specific GPIIb/IIIa receptor antagonist, had no effect on PSC might be relevant. The inhibition of PSC may be important in vitro. In contrast, we found that clopidogrel demonstrated inhibitory action on both phases of platelet activation, PSC and aggregation. This effect was enhanced when clopidogrel and aspirin were administered together. This may explain why the clopidogrel plus aspirin combination appears to be a successful anti-platelet drug combination in patients with acute coronary syndromes (ACS) (Bowry *et al.,* 2008; Patel *et al.,* 2009; Squizzato *et al.,* 2011)

Tirofiban inhibited fibrinogen-induced platelet aggregation and raised circulating levels of fibrinogen are an emerging predictor of vascular events (**Jagroop** *et al.,* 2002b; Kakafika *et al.,* 2007; Mikhailidis *et al.,* 1998a). Additionally, fibrinogen plays an important role in platelet aggregation by linking activated platelets (**Jagroop** *et al.,* 2002b; Kakafika *et al.,* 2007; Mikhailidis *et al.,* 1998a). However, although it is advantageous that tirofiban was able to reduce fibrinogen-induced platelet aggregation, it may still be a disadvantage that this drug does not inhibit PSC. This may limit the clinical effectiveness of GPIIb/IIIa receptor antagonists.

The findings described in Chapter 3 support the literature where clopidogrel was superior to aspirin in patients with PAD in terms of reducing vascular events (1996 *no authors listed*). However, clopidogrel does have several limitations, including variable absorption, drug-drug interactions and genetic factors that lead to reduced generation of the active metabolite, and a delayed onset of action (Paikin *et al.,* 2010).

The search for ADP receptor inhibitors has yielded novel ADP ($P2Y_{12}$ receptor) antagonists such as prasugrel, ticagrelor, cangrelor and elinogrel that are in current use or in various phases of clinical development (Mousa *et al.,* 2010). Moreover, it has already been shown that these ADP ($P2Y_{12}$ receptor) antagonists have advantages over clopidogrel ranging from faster onset of action, to greater and less variable inhibition of platelet function (Paikin *et al.,* 2010). However, clinical studies that are still in progress focus on this new generation of ADP receptor antagonists (parsuragrel, cangrelor and ticagrelor) as successors of clopidogrel after coronary interventions (Hall *et al.,* 2011; Kossler *et al.,* 2009).

Prasugrel (CS-747; LY-640315) is an example of a novel third-generation oral theinopyridine, that is a specific, irreversible antagonist of the platelet ADP ($P2Y_{12}$) receptor (Fletcher *et al.,* 2010; Hall *et al.,* 2011; Tagarakis, 2010). Pre-clinical and early phase clinical studies have shown prasugrel to be characterised by more potent anti-platelet effects, lower inter-individual variability in platelet response, and faster onset of activity as compared to clopidogrel (Fletcher *et al.,* 2010; Laizure *et al.,* 2010; Small *et al.,* 2010). Ticagrelor has also been shown to be superior to clopidogrel in patients with ACS (Wallentin *et al.,* 2009). Further research is needed to improve anti-platelet therapy with the aim of finding agents with favourable clinical outcome and lower bleeding risk.

This thesis also presents a new method to determine platelet cholesterol (PC) which allows a relatively rapid throughput of samples. This method was demonstrated to be reliable, reproducible quick and easy. Thus, it may be possible to assess PC in patients before and after treatment with a statin. Furthermore, PC may be a surrogate marker of tissue cholesterol. Thus, for the first time, it would be possible to assess a 'large' population for an indirect measure of 'tissue' cholesterol. For example, it will now be possible to asses PC in different populations like post-menopausal women (before and after hormone replacement therapy), the obese (before and after dieting) and diabetic patients (possibly in relation to glycaemic control). Smoking can affect high density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels (Chelland *et al.,* 2008). In turn, PC was significantly correlated with low density lipoprotein cholesterol (LDL-C) / HDL-C ratio as well as non-HDL-C cholesterol. Therefore, it may be possible to detect changes in PC after smoking cessation or when comparing smokers with non-smokers. Some of the factors considered above (e.g. diabetes, smoking and obesity) are predictors of vascular disease.

It would therefore, be of interest to investigate their effect on PC. It will also be possible to investigate the relationship between PC and indices of platelet activation (including PSC). This may be relevant because a raised PC has been linked with activated platelets (Chetty *et al.,* 1992; Labios *et al.,* 2005).

The work presented in this thesis may provide a better understanding of platelet function and how to assess it. Furthermore, potential therapeutic targets are discussed.

Suggestions for Future Work

The channelyzer method used for assessing platelet shape change (PSC) in this thesis has the advantage that it is reproducible and suited for dose and time response studies. In work done previous to this thesis, PSC has been documented in several situations of potential clinical relevance. However, the disadvantage using a Coulter ZM34 counter coupled to a C-256 channelyzer to assess PSC is that this equipment includes mercury. According to the Committee For Occupational Safety And Health (COSH) Hospital Guidance book, mercury is considered to be a toxic hazard. In order to remedy the problem of using equipment that contains mercury, we phased out the ZM34, C-256 channelyzer, and upgraded to a Multisizer 3 (Beckman Coulter). The multisizer 3 also has the advantage that it occupies less bench space than the channelyzer. The channelyzer method to asses PSC comprised of 3 linked machines (the Coulter ZM34 counter coupled to a C-256 channelyzer with the sampling stand in the middle, see figure 2.1). In contrast, the Multisizer 3 is just one machine that can be connected to a computer (see figure 7.1 below).

The Multisizer 3 is a flexible, multi-channel analyzer employing the electrical impedance method together with the state-of-the art digital pulse processing technology. It provides both platelet (or other particle) sizing and counting within an overall size range of 0.4 to 1200 μm. With the use of the very latest in digital pulse measurement technique, results may be stored either in memory, or on a disc for recall and processing on any computer or microprocessor that is compatible with the Beckman Coulter Multisizer 3 software on Microsoft Windows. One of the important factors for us is that in comparison

with the Coulter ZM-34 (C-256 Channelzyer), the Multisizer 3 uses a unique, mercury-free system to draw suspensions through the aperture at a steady rate. This technology allows for the first time, the results of an analysis to be re-assessed (e.g. up to a 100-fold increased resolution), eliminating the need to re-run the samples. A higher number of channels provide more detail about the size of the particle. Data can be displayed graphically as number, volume, mass and surface area size distribution plots, or as tables of values versus size.

Figure 7.1 Photograph of the Multisizer 3 (Beckman Coulter)

In this thesis, with the use of the channelyzer method to assess PSC, we established that both ADP receptors $P2Y_1$ and $P2Y_{12}$ were involved in the PCS phenomenon in human platelets. To our knowledge, we were the first to show an IC_{50} for the $P2Y_1$ receptor-blocker using the human PSC phenomenon. Antagonists to these ADP receptors may become useful

anti-platelet agents. The search for more improved ADP-receptor inhibitors other than clopidogrel, has yielded novel ADP ($P2Y_{12}$ receptor) antagonists such as prasugrel, ticagrelor, cangrelor and elinogrel. Since we have already done some investigations with clopidogrel, it would be of interest for us to investigate the risk -benefit profiles of these other ADP-receptor inhibitors.

There is conflicting evidence as to whether the $P2X_1$ receptor is involved in PSC. We were unable to find an effect of the $P2X_1$ receptor on PSC. This may be related to the fact that this receptor is involved with very rapid actions (1-2 s) and that it also becomes quickly desensitized. In the channelyzer method of assessing PSC, our sampling times are limited by the capacity of the operator. However, with the new Multisizer 3, it may be possible to observe these rapid changes in PSC since the method is computerised hence, it is likely to pre-set any time response. Therefore, we propose to use the new Multisizer 3 method, to investigate if the $P2X_1$ receptor is also involved in the PSC phenomenon.

We studied the effect of tirofiban, a GPIIb/IIIa receptor antagonist on fibrinogen- and agonist- induced aggregation and PSC. With the methodology used in this study it would be of interest to assess the effect of other intra-venous agents directed against the GPIIb/IIIa receptors, namely abciximab, eptifibatide, and a non-peptide mimetic, lamifiban. We found that tirofiban does not appear to have an effect at the earlier stage of platelet activation, PSC. We would like to confirm this with our latest methodology of assessing PSC, the Multisizer 3, which has the capacity to increase the resolution of an analysis by up to a 100-fold.

There have been studies comparing dual with triple therapy based on iv GPIIb/IIIa inhibitors (Moser *et al.,* 2003), aspirin, clopidogrel and/or cilostazol for preventing vascular events. Triple anti-platelet therapy was more effective than aspirin-based dual therapy on reducing vascular events in patients with acute coronary syndrome (STEMI and NSTEMI). However, insufficient data exists for patients with PAD on these drug regimes. With the technology used in this thesis, we have already investigated one GPIIb/IIIa receptor antagonist, tirofiban, on healthy volunteers, and in another study we observed the effect of clopidogrel (monotherapy and in combination with aspirin) on PAD patients. Thus, in future work, it may be possible for us to compare the effect of dual and even triple therapy on PAD patients since the 'set up' is already in place.

One of the methods developed in this thesis was for the measurement of PC. Our method proved to be simpler and less time-consuming than other methods previously used. This method should be further evaluated with a larger number of samples. In addition, we plan to assess this new method of measuring PC in hyperlipidaemic patients pre- and post-treatment with statins. It would also be of interest to use our PSC and platelet aggregation techniques concomitantly with our PC method to investigate platelets from hyperlipidaemic patients. These patients tend to demonstrate a significantly higher PC and also show that their platelets are hypersensitive to aggregation and therefore possibly shape change (an earlier phase of platelet activation).

In summary, in this thesis we have demonstrated a range of techniques that can used to assess platelet activation receptor type and release. This can be extended by future work in a clinical setting when assessing drug therapy.

APPENDIX A

Selected References Pertaining to Platelet Shape Change* (authored by I A Jagroop)

1. **Jagroop IA,** Mikhailidis DP. The effect of tirofiban on fibrinogen/agonist-induced platelet shape change and aggregation. *Clin. Appl. Thromb. Hemost*. 2008; **14**: 295-302.

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*Some of these publications overlap with the list of publications relating to platelet aggregation, below.

APPENDIX B

Selected References Pertaining to Platelet Aggregation* (authored by I A Jagroop)

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7. **Jagroop IA,** Mikhailidis DP. Mean platelet volume is an independent risk factor for myocardial infarction but not for coronary artery disease. *Br. J. Haematol*. 2003; **120**: 169-170

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*Some of these publications overlap with the list of publications relating to platelet shape change, above.

APPENDIX C

Publications List Authored by I A Jagroop of Work Presented In This Thesis*

1. **Jagroop IA,** Matsagas MI, Geroulakos G, Mikhailidis DP. The effect of clopidogrel, aspirin and both anti-platelet drugs on platelet function in patients with peripheral arterial disease. *Platelets*. 2004; **15**: 117-125. ***Related to chapter 3***

2. Matsagas M, **Jagroop IA,** Geroulakos G, Mikhailidis DP. The effect of a loading dose of clopidogrel on platelet function in patients with peripheral arterial disease. *Clin. Appl. Thromb. Hemost*. 2003; **9**: 115-120. ***Supporting paper related to findings described in chapter 3.***

3. **Jagroop IA,** Burnstock G, Mikhailidis DP. Both the ADP receptors $P2Y_1$ and $P2Y_{12}$, play a role in controlling shape change in human platelets. *Platelets*. 2003; **14**: 15-20. ***Related to chapter 4***

4. **Jagroop IA,** Mikhailidis DP. The effect of tirofiban on fibrinogen/agonist-induced platelet shape change and aggregation. *Clin. Appl. Thromb. Hemost*. 2008; **14**: 295-302. ***Related to chapter 5***

5. **Jagroop IA,** Persaud JW, Mikhailidis DP. A new rapid method to measure human platelet cholesterol: A pilot study. *Clin. Appl. Thromb. Hemost*. 2011 (*in press*) ***Related to chapter 6***

6. **Jagroop IA,** Kakafika AI, Mikhailidis DP. Platelets and vascular risk: an option for treatment. *Curr. Pharm. Des.* 2007; **13**: 1669-1683. Review. ***Related to chapter 1***

*These publications (except for reference 5) overlap with the list of publications relating to platelet shape change and/or platelet aggregation, above.

APPENDIX D:

Chemicals, Buffers & Solutions, Instruments and Apparatus

I Chemicals

Amersham International Plc, Aylesbury, UK

5-Hydroxy [side chain-2-^{14}C] tryptamine creatinine sulphate

AstraZeneca R&D, Charnwood, UK

AR-C69931MX

BDH ltd. Dagenham, UK

Acetone

Acetic anhydride

Aspirin (acetylsalicylic acid; ASA)

Hydrochloric acid (HCL)

Perchloric acid

Potassium phosphate, mono-basic

Sodium chloride

Sodium bicarbonate

di-sodium hydrogen orthophosphate, dehydrate

sodium hydroxide

tri-sodium citrate di-hydrate

trichloroacetic acid (TCA)

Coulter Electronics Ltd, Luton, UK

Standard latex particles for calibration (9fl volume)

Isoton II (a balanced electrolyte solution for blood cell counting and sizing containing: sodium sulphate 9.7 g/L; sodium chloride 4 g/L; dimethylolurea 1 g/L' procaine hydrochloride 0.1 g/L

Immunotech- Beckman Coulter, High Wycombe, Bucks, UK

5-HT Enzyme immunoassay kit

Lipha Pharmaceuticals Ltd., West Drayton, UK

Naftidrofuryl oxalate

Merk Sharp and Dohme, Hertfordshire, UK

Tirofiban (kindly donated)

Molecular Probes, Eugene Oregon, USA

TNP-ATP

R&D Systems Europe, Oxon, UK

Human soluble sP-selectin quanti-tative sandwich immunoassay

Human platelet-derived growth-factor-AB (PDGF-AB) quanti-tative sandwich immunoassay

Sanofi-Synthelabo, Paris France

Plavix (clopidogrel)

Sigma Chemicals Company Ltd., Poole, UK

Alpha,beta-methylene ATP (αβ-meATP)

N-acetylserotonin

Acetone (HPLC grade)

Adenosine diphosphate (ADP)

Arachidonic acid (sodium salt)

Calcium ionophore (A231187; CaI)

Citric acid

Cysteine hydrochloride
Dibutyryl cAMP (sodium salt)
Disodium EDTA
Fibrinogen type 1 (from human plasma, aprox. 60% protein. More than 90% of protein clottable and containing aprox. 15% sodium citrate and aprox. 25% sodium chloride)
Formaldehyde (37% aqueous; w/v)
Glutaraldehyde (25% aqueous; w/v)
Hydrogen peroxide (30% aqueous; w/v)
Human thrombin
Indomethacin
Noradrenaline bitartrate
Serotonin creatinine sulphate
tetramethylsaline
U46619 (endoperoxide/thromboxane A_2 mimetic)

Tocris Cookson Ltd, Bristol, UK
MRS 2179

II Buffers and Solutions

Citrate anti-coagulant solution
Tri-sodium citrate 3.8% w/v

Citrate-ASA anti-coagulant solution
Tri-sodium-citrate (3.8%)
ASA (10 mmol/L)

EDTA anti-coagulant solution
di-sodium EDTA (50 mmol/L)

Glutaraldehyde fixative solution

glutaraldehyde (4% w/v)

saline (150 mmol/L)

Phosphate buffer to dissolve drugs

di-sodium hydrogen orthophosphate

dehydrate (116 mmol/L)

potassium phosphate monobasic (20 mmol/L), pH 7.4

Saline

sodium chloride (150 mmol/L)

5HT assay buffer (Gow *et al.,* 1988)

citric acid (0.1 mmol/L)

sodium hydroxide (0.3 mol/L)

EDTA (1 mmol/L)

gelatin (1 g/L), pH 6.2

*All buffers and solutions were made up with double distilled water.

III Instruments and Apparatus

Balance: Sartorius research balance, Sartorius Instruments ltd, Gillingen, Germany

C-256 channelyzer: Coulter Electronics Ltd, Luton, UK

Centrifuges: Eppendorf mocro-centrifuge (5414), Anderman Ltd, UK; ICE CENTRA-7R, International Equipment Company, USA

Chronolog dual-channel optical aggregometer 540: Coulter Electronics Ltd, Luton, UK

Coulter particle counter ZM (with a 70 μm diameter sampling tube orifice): Coulter Electronics Ltd, Luton, UK

Coulter counter (MD18): Coulter Electronics Ltd, Luton, UK

Coulter ACT Diff Analyzer: Coulter Electronics Ltd, Luton, UK

Graph Pad Prism Version 4: Graph Pad Software, Inc; San Diego CA

Microplate reader: model MCC/340, ICN Flow, High Wycombe UK

Microplate shaker: Titertek, ICN Flow, High Wycombe UK

Microplate washer: Titertek, ICN Flow, High Wycombe UK

Needles: G19-21 (butterfly), Abbot Ireland, Rep. of Ireland

pH meter: Corning 140, Corning Science Products, Halstead, UK

Pipettes: Gilson pipetman and Eppendorf Repetitive pipette, Anachem, Luton, UK

Sonicator: M.S.E Soniprep, Crawley, UK

Syringes: 10 ml Sherwood Medical, Ballymoney, UK

Water Bath: Shaking water bath, Grant Instruments Ltd, Cambridge, UK

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Accumetrics Inc. VerifyNow-ASA (IIb/IIIa assay) package insert.San Diego, CA. 2007. Ref Type: Pamphlet

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